JOURNAL OF THE
NATIONAL CANCER INSTITUTE

VOLUME 8 OCTOBER 1947 NUMBER 2

INHIBITION OF CATHEPSINS OF NORMAL CALF SPLEEN AND THYMUS[1]

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INTRODUCTION

Recent studies (*1*) indicated that the activity of cathepsin preparations from the Murphy rat lymphosarcoma was inhibited by reagents which react with the sulfhydryl groups on the enzyme protein. The present studies were undertaken to determine whether this reaction is characteristic of the cathepsins of normal calf spleen and thymus.

MATERIALS AND METHODS

Enzyme Preparations

The enzyme preparations were separated from normal spleen and thymus by a method previously described in detail (*1, 2*). The most active enzyme fractions were found to be associated with the pseudoglobulin after dialysis of the precipitated globulin fraction of the tissues. Following precipitation of the pseudoglobulin by 40-percent saturation with ammonium sulfate, the protein with catheptic activity was precipitated from the filtrate by complete saturation with ammonium sulfate. Effort was made to protect any potent —SH groups on the protein by keeping the materials cold and at an acid pH. After dialysis, the preparations were lyophilized and stored in vacuum desiccators. Five spleen-cathepsin and four thymus-cathepsin preparatitons were made, and those with the most potent catheptic activity were used.

Inhibitors

The pretreatment of the enzymes with the sulfhydryl-testing reagents as inhibitors was similar to that described for the experiments with the cathepsin from the Murphy rat lymphosarcoma (*1*). The enzymes were dissolved in phosphate buffers at pH 7.0 or 7.5 and then treated with increasing amounts of inhibitor for 1 or 2 hours. Commercial *o*-iodosobenzoic acid was purified according to the method of Hellerman, Chinard, and Ramsdell (*3*). The iodacetamide was prepared in this laboratory according to the procedure of Braun (*4*). The *p*-chloromercuribenzoate was prepared according to the method of Whitmore and Woodward (*5*). The trivalent organic arsenical, 3-amino-4-hydroxyphenyldichlorarsine hydrochloride, was obtained from the Laboratory of Biologics Control, National Institute of Health. The concentrations of inhibitors are given in micromoles ($M \times 10^{-6}$) per milligram enzyme in 1 cc. buffer. The percent of inhibition was calculated by using the optimal activity at pH 3.5, without pretreatment, as 100 percent. The total inhibitions are given since it could not be determined how much inhibition was caused by the alkaline reaction of the digest in the presence of the inhibitors.

Determination of Catheptic Activity

The catheptic activities were determined by a modification of Anson's technique (*6*), in which the liberated tyrosine color was measured after hemoglobin was hydrolyzed by 1 mg. of enzyme for 90 minutes at 39° C., and at a pH of 3.5. The nonprotein nitrogen was determined in the same trichloracetic acid filtrates as the tyrosine color by the method of Koch and McMeekin (*7*). The most active spleen-cathepsin preparations liberated 3.0 mg. of nonprotein nitrogen and 1.25 mg. of tyrosine color equivalent under these conditions. The most active thymus-cathepsin preparations liberated 1.47 mg. of nonprotein nitrogen and 0.68 mg. of tyrosine color equivalent.

RESULTS

The reagents which are known to react with the —SH groups of proteins fall into three classes: (1) Those which oxidize the —SH groups; (2) those which form reversible mercaptans or arsenites; and (3) those which alkylate the —SH groups. Since all these reactions require a neutral

[1] Received for publication July 18, 1947.

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or alkaline medium, it was essential to know the effect of alkali alone upon the enzymes. The effect of pretreatment of the enzymes with pH 7.0 and 7.5 phosphate buffers for 1 hour was an inhibition of from 4 to 20 percent. Two hours' pretreatment at pH 7.5 caused up to 30 percent inhibition. With the more concentrated solutions of *p*-chloromercuribenzoate, a pH of 8.4 was necessary for solution, but the inhibitory effect of pretreatment at this pH was no greater than at pH 7.5.

Since the —SH groups of proteins may be sensitive to oxidation by air at an alkaline pH, the effect of aeration for 1 hour on alkaline solutions of these enzymes was tested. The inhibitory effect was slightly more than that caused by the pH 7.5 buffer alone.

Titration of the —SH Groups in Cathepsins

The total sulfhydryl groups in the spleen- and thymus-cathepsin preparations were titrated according to the method described by Hellerman, Chinard, and Deitz (*8*), which was used in our earlier study of the cathepsin of rat lymphosarcoma (*1*). To a solution of 10 mg. of the dried cathepsin preparations in 0.5 ml. M/15 phosphate buffer at pH 7.0 were added 1.5 ml. water, 0.5 ml. M buffer at 7.0, and 1 gm. neutral guanidine hydrochloride. After 30 minutes' standing under nitrogen at room temperature, 3 ml. of 0.00200N sodium iodosobenzoate was added. After 2 minutes, 0.5 gm. potassium iodide dissolved in 3 ml. N hydrochloric acid was added, and the iodine was titrated with 0.00202N sodium thiosulfate; from 2.40 to 2.52 ml. thiosulfate was required. These titrations indicated the presence of 1.16 to 0.91 micromoles of titratable —SH in 10 mg. of enzyme preparation. The same values were given when guanidine hydrochloride was not added, showing that the enzyme-proteins were probably denatured in the process of preparation.

Inhibitory Effects of the Pretreatment of the Cathepsins with Sulfhydryl-Testing Reagents

p-Chloromercuribenzoate

p-Chloromercuribenzoate, which has been regarded as the most sensitive sulfhydryl-testing reagent (*8–10*), was the most potent inhibitor of both the spleen and the thymus cathepsins. The spleen-cathepsin preparations were all more sensitive to the mercury reagent than were the thymus cathepsins (fig. 1). When treated for 1 hour at pH 7.0 with 3 micromoles per milligram enzyme, the thymus cathepsin was inhibited only 15 percent.

After the enzymes were exposed to low concentrations of the *p*-chloromercuribenzoate for 15 minutes, the inhibitory effects could be reversed by adding excess glutathione. Thus, spleen cathepsin III was inhibited 37 percent by pretreatment

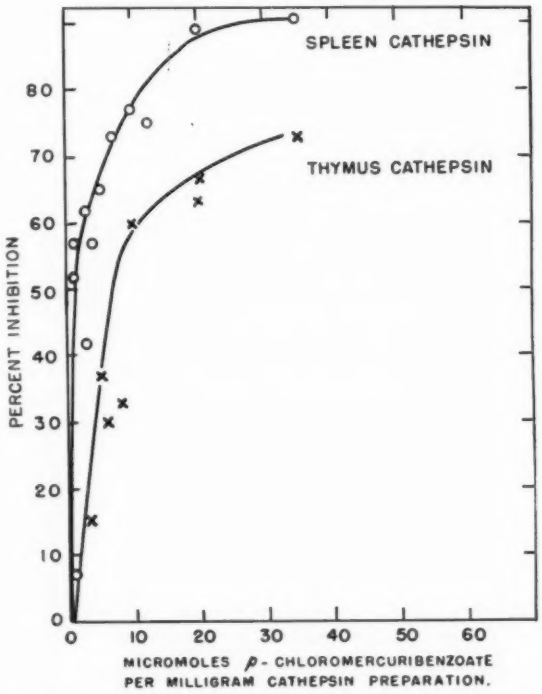

Figure 1.—Spleen and thymus cathepsins treated with increasing quantities of *p*-chloromercuribenzoate for 1 hour at pH 7.0. Inhibition was determined by the decrease in nonprotein nitrogen liberated when the treated enzymes hydrolyzed hemoglobin for 90 minutes at pH 3.5 and 39° C.

for 15 minutes with 2 micromoles of the mercury reagent, and after the addition of 20 equivalents of glutathione, the inhibition was reduced to 14 percent. Both thymus and spleen cathepsins could be completely reactivated with glutathione after pretreatment with 0.5 to 1 micromole of this reagent. When these enzymes, especially the spleen cathepsin, were in contact with the *p*-chloromercuribenzoate at pH 8.4 for 2 hours, considerably more proteinase activity was lost (table 1). The inhibitory effect upon the liberation of tyrosine color liberated by the spleen cathepsin was much less than upon the liberation of nonprotein nitrogen under these conditions. In most of the inhibitory experiments, the percent of inhibition of tyrosine color and the percent of inhibition of the nonprotein nitrogen liberated were parallel.

Sodium Iodosobenzoate

The maximum inhibition of 47 percent was obtained with the most active spleen cathepsins when pretreated with 4 micromoles of this inhibitor per milligram of enzyme for 1 hour at pH 7.0. Increasing amounts of the sodium iodosobenzoate did not

TABLE 1.—*Inhibition of spleen and thymus cathepsins by pretreatment with* p*-chloromercuribenzoate for 2 hours at pH 8.4*

Enzyme	Pretreatment of enzyme, concentration of inhibitor	Hydrolysis	
		Tyrosine liberated	Protein N hydrolyzed
		Inhibition	Inhibition
	Micromoles	*Percent*	*Percent*
Spleen cathepsin III		4.6	9.2
	3	28	42
	5	31	66
	7	34	73
		8.3	8.3
Thymus cathepsin III	3	16	15
	6	27	30
	8	24	33

increase this inhibition. The thymus cathepsins were less sensitive to this inhibitor since 20 micromoles per milligram of enzyme caused only 33-percent inhibition in the liberation of tyrosine.

IODACETAMIDE

The thymus cathepsins were also less sensitive to inhibition by iodacetamide than were the spleen cathepsins (fig. 2). While 5 micromoles caused an inhibition of 50 percent in the hydrolysis by the spleen cathepsin, 25 micromoles were needed to inhibit the thymus cathepsin 45 percent.

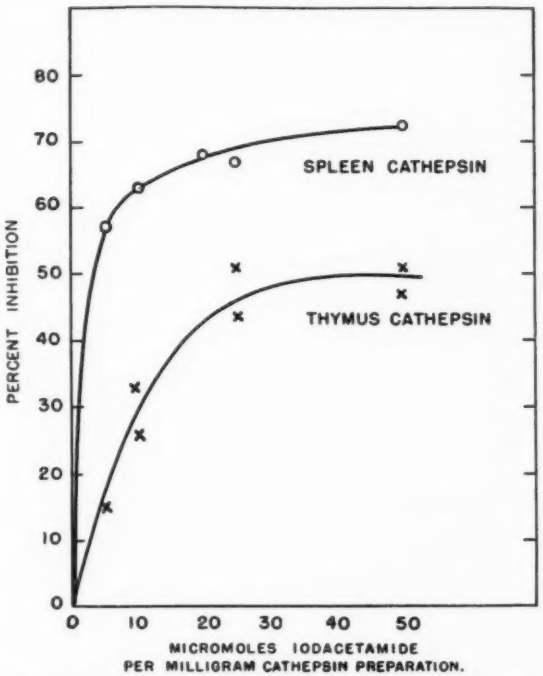

FIGURE 2.—Spleen and thymus cathepsins treated with increasing quantities of iodacetamide for 1 hour at pH 7.0. Inhibition was determined by the decrease in nonprotein nitrogen liberated when treated enzymes hydrolyzed hemoglobin for 90 minutes at pH 3.5 and 39° C.

3-AMINO-4-HYDROXYPHENYLDICHLORARSINE HYDROCHLORIDE

In amounts up to 20 micromoles per milligram of both the spleen and the thymus cathepsins, 3-amino-4-hydroxyphenyldichlorarsine hydrochloride caused no more inhibition than did pH 7.0 buffer alone.

DISCUSSION

The spleen- and thymus-cathepsin preparations were less sensitive to the sulfhydryl-testing reagents than was the rat lymphosarcoma cathepsin previously studied. Although the titrations for the —SH groups on the protein of these cathepsins gave the same range of values as were given by the rat-tumor cathepsin, over 10 times as much of the mercury reagent was needed to cause 50-to-70-percent inhibition. The molar equivalent of the *p*-chloromercuribenzoate needed to combine with the —SH groups on the spleen cathepsin caused an inhibition of only 10 to 20 percent. This inhibition could be reversed by the addition of excess glutathione.

Since the cathepsins lost more activity when exposed to the *p*-chloromercuribenzoate for 2 hours at pH 8.4, it is possible that the —SH groups essential for the activity were not readily available for mercaptide formation. It is also possible that some of the enzymic components of the cathepsin are not dependent upon substituent —SH groups for the hydrolysis of certain of the many different peptide linkages in the hemoglobin molecule. This hypothesis may be related to the observation of Fruton, Irving, and Bergmann (*11*) that cysteine activation was required by some of the components of beef-spleen cathepsin to hydrolyze certain synthetic peptides, while some components were able to hydrolyze other synthetic peptide bonds without activation.

The spleen cathepsin was much more readily inhibited by the mercury reagent: the alkylating reagent, iodacetamide; and the oxidizing reagent, iodosobenzoate, than was the thymus cathepsin. This difference would indicate that proportionately more of the —SH groups of the spleen cathepsin-protein were essential for the hydrolysis of the peptide bonds of hemoglobin.

SUMMARY

Cathepsin preparations from normal calf spleen and thymus were treated with the following sulfhydryl-testing reagents: *p*-chloromercuribenzoate, sodium iodosobenzoate, iodacetamide, and 3-amino-4-hydroxyphenyldichlorarsine hydrochlo-

ride. The effects of these pretreatments upon the ability of the enzymes to hydrolyze hemoglobin were tested. Only the last-named of these reagents was without some inhibitory effect.

The cathepsins prepared from calf spleen were more active in hydrolyzing hemoglobin and were also more sensitive than the thymus cathepsins to the sulfhydryl-testing reagents.

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SUBCUTANEOUS SARCOMAS IN MICE IMPLANTED WITH HYDROCARBON-CHOLESTEROL PELLETS[1]

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INTRODUCTION

Previous communications (*1*, *2*) analyzed data dealing with the responses of mice to measured quantities of three carcinogenic hydrocarbons dissolved in oily media. The primary purposes of these studies were twofold: (1) To supply information on the nature of the relationship between dose of subcutaneously injected carcinogenic hydrocarbons and the local tumor responses which they elicit in test mice. These data are essential for appropriate statistical procedures that may be employed in analyses of the biologic results and in testing the significance of differences obtained under various experimental conditions. (2) To compare the relative carcinogenic potencies and relative speeds of action of 20-methylcholanthrene, 3, 4-benzpyrene and 1, 2, 5, 6-dibenzanthracene. Such data are part of the information necessary for the eventual elucidation of their mode of action in eliciting the neoplastic response.

The present experiment extends the studies by presenting data obtained with the three hydrocarbons dissolved in cholesterol and administered in the form of solid pellets. This useful method, originated by Shear (*3*), should be applicable in investigations in which induced tumors are desired and in which removal of the bulk of the tumor-inciting agent is required (*4*).

EXPERIMENTAL PROCEDURE

The corrected melting points of the three hydrocarbons used were: methylcholanthrene, 179.7°–180.2° C.; dibenzanthracene, 267.3°–267.7°; and benzpyrene, 178.2°–178.9°. The cholesterol employed was obtained from the spinal cord of cattle and had a melting point of 149°–150° C. The hydrocarbons came from the same batches used previously (*2*).

The hydrocarbon-cholesterol pellets were prepared by a method described by Shear (*3*). Appropriate amounts of the three hydrocarbons were mixed with cholesterol. The mixtures were heated to melting in a beaker immersed in an oil bath and drawn by means of a metal plunger into thick-walled glass tubes with an aperture of 1 mm. The glass tubes and the plungers were not lubricated with oil. The resultant solid sticks that were extruded were cut into lengths of approximately 2 mm. The pellets weighed 1.8 to 2.6 mg., the great majority falling within the 1.9–2.1-mg. range.

In this manner, cholesterol pellets containing 0.1, 0.25, 0.5, 1.0, 5.0, 10.0, and 25.0 percent of the respective three hydrocarbons were obtained. The average total quantities of the carcinogenic hydrocarbons in the pellets were, therefore: 0.002, 0.005, 0.01, 0.02, 0.1, 0.2, and 0.5 mg., respectively.

The pellets were introduced subcutaneously by means of a trocar into the right upper flank of male mice of the C3H strain (Andervont subline). Each mouse received a single pellet. The animals were 2 months old at the time of injection and weighed 18 to 24 gm., the majority falling within the 20–22-gm. range. The first lot of mice received injections on September 1, 1943, and additional lots were added during the following 12 months. Each lot was divided at random into 21 groups for injection with the respective doses and hydrocarbons.

The animals were kept in wooden boxes, five to eight per box, with wood shavings as bedding. They were maintained on unlimited supplies of water and Purina dog chow and were individually identified.

Examination by palpation of the mice was carried out weekly beginning 6 weeks after injection. Mice with subcutaneous nodules thought to be early tumors were isolated at each examination. A progressively growing tumor, proved by subsequent histologic appearance to be a malignant neoplasm, was listed as having appeared on the date on which the nodule was first recorded. The interval between injection and appearance of the tumor was considered the latent period.

Of the 676 mice originally employed, 645 contributed to the final analysis of the results. Thirty-one were listed as missing or died during the first 3 months of the experiment and were discarded from the results.

The subcutaneous tumors were fixed in Zenker's fluid and embedded in paraffin. The sections were stained with hematoxylin and eosin for histologic examination. A total of 168 tumors at the site of injection was observed. No sections were obtained on 2 rapidly growing subcutaneous masses (elicited with 0.5 percent dibenzanthracene and 25.0 percent benzpyrene pellets, respectively) but were accepted in the data as tumors.

RESULTS

The 2-mg. hydrocarbon-cholesterol pellets were well tolerated by the mice. There were no cases of ulceration at the site of implantation. The presence of the pellets was easily ascertained by palpation, and in almost all cases the pellets remained in situ or close to the location into which they were injected. The pellets were recovered at autopsy in

[1] Received for publication July 14, 1947.

all animals accepted in the results. No obvious great reduction in size of the pellets, or softening of the pellets was noted. Determinations by Shear and Lorenz (*5*) indicate that most of the carcinogen is recoverable from pellets that have remained in the subcutaneous tissue of mice for protracted periods. Although no similar determinations were made in this experiment, it can be stated with some assurance that no more than 10 percent of the carcinogen was absorbed or otherwise disappeared from the pellets.

Of the 166 tumors examined histologically, all were sarcomas of the type described previously (*6*). Except for the absence of oil cysts, they were indistinguishable from the sarcomas obtained with the hydrocarbons dissolved in tricaprylin. The only unusual tumor was a sarcoma with areas of bone which arose 17 months following implantation of a 10 percent dibenzanthracene pellet.

No epithelial tumors were observed. This contrasts with the occurrence of carcinomas in 4 of 415 tumors of the previous investigation in which the hydrocarbons were dissolved in 0.25 cc. of tricaprylin (*6*).

All tumors evoked in this experiment were single masses, in contrast with the occurrence of multiple tumors in C3H male mice injected with the hydrocarbons in tricaprylin. The incidence of multiple tumors appears to be related to the volume and spread of the vehicle in which the carcinogen is introduced:

Hydrocarbons in 0.25 cc. tricaprylin, 22 of 415 tumors, or 5.1 percent.
Hydrocarbons in 0.1 cc. tricaprylin, 2 of 63 tumors, or 3.2 percent
Hydrocarbons in 2-mg. pellets, 0 of 168 tumors, or 0 percent.

The findings are evidence toward a conclusion that multiple tumors and perhaps the mixed tumors are due primarily to the area with which the injected carcinogen comes in contact.

Table 1 summarizes the results of the experiment. Analyses [2] were made to determine the variability of the biologic responses and to determine the trends of the various group responses with different concentrations of the hydrocarbons. Most of the biomathematical procedures involved in these analyses are discussed by Fisher (*7*), and their applications to results with carcinogenic hydrocarbons were described elsewhere (*1*, *2*).

In considering animals that did not develop tumors and were lost by death during the course of the experiment, the method of correction previously proposed by Bryan and Shimkin (*1*) was used. The more involved technique of Lea (*8*) was also applied, but it gave no improvement over the method of Bryan and Shimkin. It is possible that the life-table methods used by Irwin and Goodman (*9*) would be preferable to either of these procedures when large numbers of animals are involved. The corrections produced useful gradations of results obtained with methylcholanthrene and with benzpyrene. The low responses to dibenzanthracene made such corrections inapplicable, and in this instance corrected totals for deriving tumor incidence were obtained by omitting all animals that died before the appearance of the first tumors, at 7 months after injection.

Analysis of the relation of the concentration of hydrocarbons in the pellets to the incidence of tumors showed that responses to methylcholanthrene were more frequent than to benzpyrene or dibenzanthracene at levels of 0.25 percent or higher. Methylcholanthrene and benzpyrene gave the appearance of similar response, i. e., the slopes of regression of probits on log dose were parallel within the limits of variability. Comparison of the data with results obtained when these carcinogens were dissolved in tricaprylin (*2*) showed no significant difference. This does not imply that such differences did not exist, but merely that the variability of the responses was too great for the demonstration of statistically significant differences.

The TD_{50} doses for methylcholanthrene and for benzpyrene in this investigation were between 0.01 and 0.02 mg. and 0.02 and 0.1 mg., respectively. This is in agreement with the TD_{50} doses for these hydrocarbons dissolved in tricaprylin, 0.02 and 0.1 mg., respectively. No particular significance can be attached to this finding, however. In the cholesterol pellets, 90 percent or more of the carcinogenic material can be recovered from the pellets at the termination of the experiment (*5*), so that the actual carcinogenic dose is no more than one-tenth of the original amount incorporated in the pellets.

The response to dibenzanthracene was markedly influenced by its incorporation in cholesterol. The response was definitely different from the response to the other two carcinogens and from the response to dibenzanthracene when it was dissolved in tricaprylin. The slope of the regression of incidence on dose was significantly depressed. Despite the low incidence of tumors at each concentration level, dibenzanthracene elicited tumors with lower concentrations than did either methylcholanthrene or benzpyrene. This finding is in general agreement with previous results (*2*) and with the work of Shear and Lorenz (*5*) which suggest that at low doses dibenzanthracene may exhibit greater carcinogenic potency than do methylcholanthrene or benzpyrene.

The relation of concentration of the hydrocarbons in the pellets to the latent periods was studied, and the regressions were found to be not significantly different from zero with each of the three materials. Larger groups of animals are

[2] Mr. D. G. Delhi, of the National Cancer Institute, made the analyses.

TABLE 1.—*Subcutaneous sarcomas in male C3H mice implanted with hydrocarbon-cholesterol pellets*

[Numerator-mice with tumors
Denominator-mice dying without tumors]

Concentration of hydrocarbon in pellet (percent)	Hydrocarbon in pellet	Tumors and deaths without tumors, in months after implantation of pellets																Total mice	"Corrected" total mice [1]	Total tumors	Percentage of tumors	Average latent time
		3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24+					
	Mg.																	*Number*	*Number*	*Number*		*Months*
25.0	0.5	1/0	4/3	4/0	3/0	.....	2/0	.....	2/0	.....	1/0	1/1	.....	.....	.....	.....	.....	22	19.4	18	92.8	6.1
10.0	.2	2/0	4/0	6/0	1/0	1/0	.....	1/0	.....	1/1	.....	0/1	.....	.....	0/1	.....	0/1	20	20.0	16	80.0	5.1
5.0	.1	1/0	5/0	4/0	3/0	1/0	.....	.....	1/0	1/0	1/0	.....	.....	0/4	.....	.....	.....	21	21.0	17	81.0	5.6
1.0	.02	.....	3/0	2/0	3/0	3/0	2/0	0/1	.....	0/1	.....	0/2	0/1	0/2	0/1	.....	.....	21	20.8	14	66.7	5.9
.5	.01	.....	.....	1/1	4/0	3/2	1/0	.....	0/1	1/2	1/0	0/4	0/3	0/5	0/8	.....	0/1	38	36.3	11	30.3	7.0
.25	.005	.....	0/1	1/0	1/0	2/1	1/0	1/0	0/1	1/2	0/2	0/3	0/1	0/4	0/9	0/4	0/5	40	38.0	7	18.4	7.7
.1	.002	.....	.....	0/1	.....	.....	.....	.....	.....	.....	0/2	0/5	0/7	0/4	0/10	0/8	0/3	39	38.5	0	.....	.....
	3,4-BENZPYRENE																					
25.0	0.5	1/0	3/0	5/0	3/0	1/0	.....	1/0	0/1	1/1	.....	.....	0/1	0/1	.....	.....	0/1	20	19.9	15	75.3	5.2
10.0	.2	.....	3/0	3/1	5/0	2/0	.....	.....	.....	0/1	3/1	0/1	.....	0/2	.....	.....	0/1	23	22.7	16	70.5	6.1
5.0	.1	.....	4/0	2/0	1/1	2/0	2/0	.....	0/1	1/0	1/0	0/1	.....	0/1	0/1	.....	0/2	20	19.4	13	67.0	6.1
1.0	.02	.....	.....	1/0	0/1	0/1	.....	.....	2/0	.....	0/1	0/1	0/6	0/2	0/4	0/3	0/4	26	25.2	3	11.9	8.0
.5	.01	.....	1/0	1/0	.....	2/0	1/0	.....	.....	.....	1/0	0/3	0/2	0/9	0/13	0/3	0/4	40	40.0	6	15.0	6.6
.25	.005	.....	.....	.....	0/1	.....	0/1	.....	.....	.....	.....	0/6	0/5	0/2	0/11	0/8	0/6	40	39.4	0	.....	.....
.1	.002	.....	.....	.....	0/1	0/1	.....	.....	.....	.....	0/1	0/7	0/2	0/10	0/7	0/7	0/4	40	39.3	0	.....	.....
	1,2,5,6-DIBENZANTHRACENE																					
25.0	0.5	.....	0/1	.....	.....	.....	0/1	.....	0/1	0/2	2/2	2/2	1/0	2/5	0/1	0/3	0/1	26	[2] 25	7	[2] 28.0	14.2
10.0	.2	.....	.....	.....	.....	.....	1/0	.....	.....	0/2	1/0	1/0	0/5	3/5	0/2	0/3	0/4	27	27	6	22.2	14.1
5.0	.1	.....	.....	.....	.....	.....	2/1	1/2	1/0	.....	0/2	.....	0/3	0/6	1/5	0/4	0/1	29	29	5	17.2	10.1
1.0	.02	.....	.....	.....	.....	.....	.....	0/1	.....	0/2	1/0	0/4	1/3	2/9	0/2	0/3	0/3	31	31	4	12.9	15.3
.5	.01	.....	0/1	0/2	.....	1/0	1/0	.....	1/0	0/2	1/2	2/3	1/2	0/3	0/11	0/4	0/4	41	[2] 38	7	[2] 18.4	10.6
.25	.005	.....	.....	.....	0/1	.....	0/2	1/0	.....	0/4	1/4	0/3	0/6	0/3	0/8	0/4	0/5	42	[2] 41	2	[2] 4.9	11.3
.1	.002	.....	.....	0/1	0/1	.....	1/0	.....	.....	0/1	.....	0/3	0/2	0/7	0/15	0/3	0/5	39	[2] 37	1	[2] 2.7	8.0

[1] Compare Bryan and Shimkin (1).
[2] Not counting mice surviving less than 7 months.

necessary to establish lack of relationship between latent period and concentration of hydrocarbon in cholesterol pellets. As with the incidence-response data, all that can be said is that the variability of the latent-period responses was too great to establish a significant regression. However, in contrast with the data obtained when the carcinogens were dissolved in tricaprylin, the findings suggest that localization of the hydrocarbons by means of cholesterol may obliterate differences in latent-period responses at different concentrations of the carcinogens.

Since there was no significant regression of latent periods on dose, the latent-period data for all dose groups of each hydrocarbon were combined. The average latent periods for methylcolanthrene and for benzpyrene were found to be the same, 6.0 months. The average latent period for dibenzanthracene was significantly longer, 12.5 months. If these data are compared with the average latent periods obtained with the TD_{50} amounts of the three hydrocarbons dissolved in tricaprylin, it is found that the latent period is significantly longer only in the case of dibenzanthracene. As with the incidence data, therefore, and within the wide limits of variation, incorporation of the three hydrocarbons in cholesterol as contrasted with tricaprylin as a vehicle seems to alter the response to dibenzanthracene more markedly than to either methylcholanthrene or benzpyrene.

The data of the present investigation are in general agreement with previous work with hydrocarbon-cholesterol pellets implanted subcutaneously in mice. Shear and Lorenz (*5*) used cholesterol pellets weighing 12.2 to 48.1 mg. and containing from 0.001 to 1 percent dibenzanthracene, in strain A mice. Shear and Ilfeld (*10*) reported results with pellets weighing 7 to 60 mg. and containing 0.001 to 5 percent of methylcholanthrene, benzpyrene, and dibenzanthrene, in strain dba mice. Tumors were not obtained with pellets containing 0.1 percent or less of the hydrocarbons, with the exception of 1 sarcoma elicited in 18 strain A mice implanted with a 0.001 percent dibenzanthracene pellet. The data are insufficient and inapplicable for quantitative comparison with results in C3H mice used in this experiment. Andervont and Shear (*4*), using 5 percent methylcholanthrene-cholesterol pellets weighing 12 to 15 mg., elicited sarcomas in all of 15 C3H mice at an average latent period of 3.3 months. The higher incidence and the shorter average latent time may be a function of the larger surface area exposed

to the tissues of the animal than with 2-mg. pellets used in this investigation.

One of the results of this experiment is to indicate again the need for large groups of animals in many problems in cancer research. If the number of mice had been doubled for the methylcholanthrene group, the results might have been amenable to definite interpretation. Still more animals would have been required to interpret definitely the data with benzpyrene and with dibenzanthracene.

SUMMARY

Male mice of strain C3H were implanted subcutaneously with single pellets of cholesterol weighing 2 mg. and containing from 0.1 to 25.0 percent of 20-methylcholanthrene, 3,4-benzpyrene, or 1,2,5,6-dibenzanthracene; 168 sarcomas at the site of implantation were produced in 645 mice. Data relative to sarcoma induction at various concentration levels are presented.

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VASCULAR REACTIONS OF NORMAL AND MALIGNANT TISSUES IN VIVO. II. THE VASCULAR REACTION OF NORMAL AND NEOPLASTIC TISSUES OF MICE TO A BACTERIAL POLYSACCHARIDE FROM SERRATIA MARCESCENS (BACILLUS PRODIGIOSUS) CULTURE FILTRATES[1,2]

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INTRODUCTION

It has been known since the experimental work of Gratia and Linz (*1*), confirmed by Shwartzman and Michailovsky (*2*), that intraperitoneal injection of certain bacterial products results in hemorrhage, followed by necrosis in transplanted tumors. Various phases of this problem have been studied in this Institute since 1932 by Andervont and Shear. The earlier work has been reviewed by Shear (*3*), Shwartzman (*4*), and Andervont (*5*). Shear and co-workers (*6–10*) have obtained a hemorrhage-producing polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*). In presenting their results and in discussing earlier work in this field, they have emphasized the need for information as to the mechanism through which these products damage tumors.

A method for the direct microscopic observation and measurement of some effects of hemorrhage-producing agents on transplanted tumors was provided by the development of quantitative in vivo techniques (*11–13*) for the study of vascular development in tissue transplants in mice. In the experiments reported herein, these methods have been applied in seeking information regarding the action of the bacterial polysaccharide from *Bacillus prodigiosus* on the peripheral vascular bed, including the capillary blood supply of a normal tissue (striated muscle); the action on capillaries in transplanted tumors; the sequence of events leading to hemorrhage and necrosis; and the pattern of regrowth following hemorrhage.

MATERIALS AND METHODS

The material used was a polysaccharide fraction (P–3) from *Bacillus prodigiosus*.[3]. Intraperitoneal injection was made of the material into mice carrying transparent chambers. In this study amounts of 0.02 and 0.2 mg. were used routinely as small and large doses, respectively, as suggested by Shear. These concentrations were based on Shear's finding (*10*) that the L. D. 50 was 0.1 mg. for control mice of strain A and albino backcross ABC, and 0.015 mg. for mice with primary tumors. Such minimal doses as 0.0001 mg., which Shear found to produce detectable hemorrhage in tumors, were not used in this study.

The general procedure was as follows: Tumor tissues were transplanted into transparent chambers at the time of insertion of the chamber. Quantitative measurements were made of the relative amount of vascular tissue (in terms of volume percentage of the total tissue observed) [4] prior to and at frequent intervals following the injection of the polysaccharide according to the method described by Chalkley (*12*). Photographic records were obtained of significant stages in the changes that occurred. Tissues were fixed in Zenker-formol and stained with hematoxylin and eosin, either at the end of the transparent-chamber observations or at the death of the mouse from progressive growth of the tumor.

The transparent-chamber preparations used allowed microscopic observation and measurement of the vascular components of subcutaneous tissues as well as observation and measurement of the capillaries in the layer of striated muscle which lies directly beneath the mica cover slip. These vascular components were measured separately, the arteries and veins of the subcutaneous tissue being taken as the connective-tissue vascular level, while the capillary vascular level was obtained by an independent count on capillaries lying between the muscle bundles. Figure 1 shows these vessels as seen in vivo, the capillaries of the striated-muscle layer appearing as fine parallel lines and the arterioles and venules in a deeper plane and irregularly distributed.

RESULTS

PERIPHERAL CIRCULATION

After intraperitoneal injection of the polysaccharide, progressive slowing of the venous and arterial circulation, with apparent increase in circulating leukocytes, was observed within 1 to 2 hours. Venous engorgement was a prominent feature of the response to the higher dose (0.2 mg.), but this response was slight at the lower dose (0.02 mg.) (table 1). Capillaries of the striated-muscle

[1] Received for publication July 22, 1947.
[2] Presented in part before the American Association for Cancer Research, Atlantic City, N. J., March 11, 1946.
[3] This material was obtained from Dr. M. J. Shear, of the National Cancer Institute.

[4] In this method, a microscope fitted with an eyepiece arranged to show a fixed pattern of points in the focal plane is used. Repeated presentation of this pattern to the tissue is made, and the visual contacts of the points with the tissue to be measured are recorded. The ratio between the number of contacts with the tissue measured and the total chances of contact in the volume covered is directly proportional to the volume ratios between the tissue and that total volume. The absolute volume values are not needed. The percentage of all possible hits that fall on vessels is taken as the measure of the vascular level in the tissue examined. Each point on the curves represents a value for the vascular levels obtained by such measurements of 100 microscopic fields.

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layer, however, showed significant decreases in vascular level at either dose (table 2). Observation revealed progressive sluggishness of blood flow, concentration of red cells, and stasis of flow in the capillaries. Neither hemorrhage nor necrosis was observed in the striated muscle, but with the development of decreased blood supply there was a cessation of the characteristic intermittent muscular contraction. If the mouse survived, muscle function returned as the capillary bed returned to normal levels. The quantitative data on seven experiments are given in table 2. Figure 2 shows graphically the measurements in a typical experiment.

Photographic evidence was obtained that the progressive development of stasis resulted in edema of the subcutaneous tissues. Figure 1, *A*, taken prior to injection of bacterial polysaccharide, shows that when the parallel capillaries of the muscle layer are in sharp focus, the underlying arterioles and venules of the subcutaneous tissue are included in the focal plane of the lens used. Figure 1, *B*, of the same field at approximately the same focal plane, 4 hours after injection, shows the separation of these arteriole and venule components from the capillaries, as indicated by their failure to be included in the same depth of field as in figure 1, *A*.

Transplanted Sarcomas

A total of 11 sarcoma transplants was used, of which 4 were from sarcoma 37, and the remainder from sarcomas D, H, J, and L.[5] As indicated in table 3, experiments from 1 to 7 were carried out in chambers in which the tumor was confined between 2 layers of mica. The remainder were in single-layer chambers in which the tumors were free to expand. All reacted similarly to the injection of the polysaccharide. Slowing of circulation in the peripheral vascular bed and in the tumor capillaries was noted within 1 to 2 hours after injections. This was accompanied by increased concentration of red blood cells and finally stoppage of circulation, leading to partial or complete occlusion of tumor capillaries. Measurements showed decreases in tumor vascular levels as early as 1 hour after injection of the polysaccharide. Hemorrhage occurred in 8 of the tumors after 3 or 4 hours of progressively developing vascular occlusion. Decreased vascular levels were attained, however, even in the 3 tumors in which hemorrhage was not seen. The low point of the vascular levels was reached in approximately 4 hours after injection. Necrosis became apparent in about 18–24 hours as an opacity of the central portion of the tumor. In two instances, areas of necrosis became apparent in the absence of hemorrhage, but only after prior occlusion and decreased vascular level. Even in instances in which there was apparently complete necrosis of the tumor, the surviving cells at the periphery of the tumor retained the capacity to elicit a new vascular supply,

[5] Obtained from Dr. Wilton R. Earle, of the National Cancer Institute.

Table 1.—*Effect of bacterial polysaccharide on vascular levels in subcutaneous connective tissue (arterioles and venules)*

Experiment No.	Strain	Dose	Vascular level									Mortality
			Starting hour 0	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	24 hours	48 hours	
		Mg.										
1	C	0.15	25	.....	29	38	.....	.....	.....	.....	.....	Died.
2	C	.10	22	26	28	30	30	.....	.....	.....	.....	Do.
3	C	.02	30	28	30	.....	31	.....	30	27	31	Recovered.
4	C3H	.20	22	28	30	.....	29	34	25	.....	.....	Died.
5	C3H	.20	28	31	31	32	33	.....	27	.....	.....	Do.
6	C3H	.20	24	28	29	36	30	31	29	29	25	Recovered.
7	C3H	.02	22	25	.....	.....	.....	.....	22	.....	27	Do.
8	C	.1	24	25	28	.....	27	24	23	24	.....	Do.
9	C	.4	23	23	31	.....	.....	.....	26	.....	.....	Do.
10	C	.2	24	28	25	38	.....	.....	.....	27	30	Do.
11	C	.2	26	.....	35	.....	.....	31	.....	40	.....	Died.

Table 2.—*Effect of bacterial polysaccharide on vascular levels in capillaries of striated muscle*

Experiment No.	Strain	Dose	Vascular level										Mortality
			Starting hour 0	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	6.5 hours	24 hours	48 hours	
		Mg.											
1	C	0.15	28	31	22	18	20	.....	.....	.....	.....	.....	Died.
2	C	.10	29	37	31	25	21	.....	.....	.....	.....	.....	Do.
3	C	.02	33	35	32	24	25	21	.....	25	.....	.....	Recovered.
4	C3H	.20	23	13	.....	11	15	14	.....	13	.....	.....	Died.
5	C3H	.20	27	22	19	14	15	16	.....	15	.....	.....	Do.
6	C3H	.20	34	24	16	18	14	14	.....	15	30	27	Recovered.
7	C3H	.02	29	.....	19	.....	5	10	.....	6	40	.....	Do.

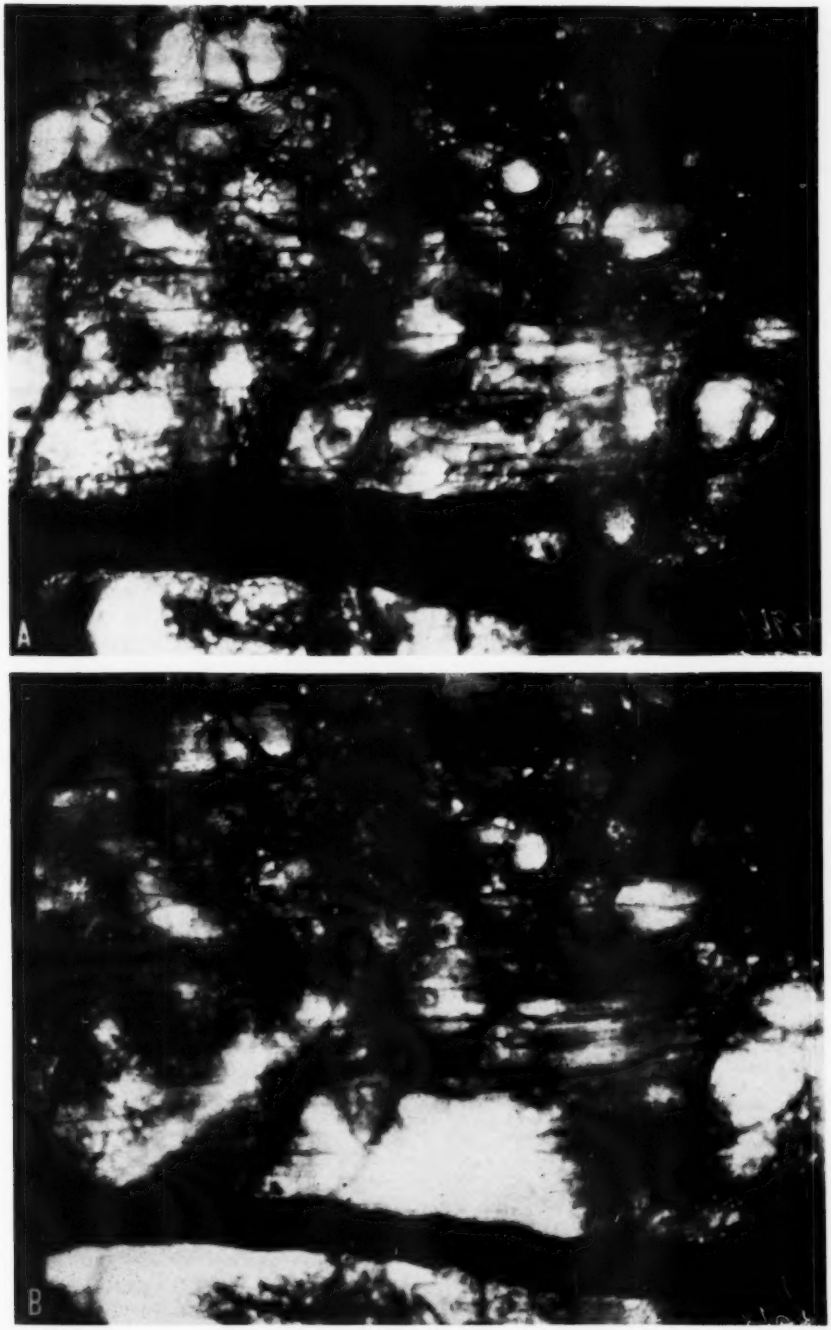

FIGURE 1.—*A*, Capillaries of striated-muscle layer, showing underlying arterioles and venules of subcutaneous tissue in same focal plane prior to injection of bacterial polysaccharide. ×150 (Reduced one-half). *B*, Same field as *A*, 4 hours after injection, showing optical evidence of edema as indicated by separation between capillaries in muscle layer and underlying vessels in subcutaneous tissue, which appear indistinct. ×150. (Reduced one-half.)

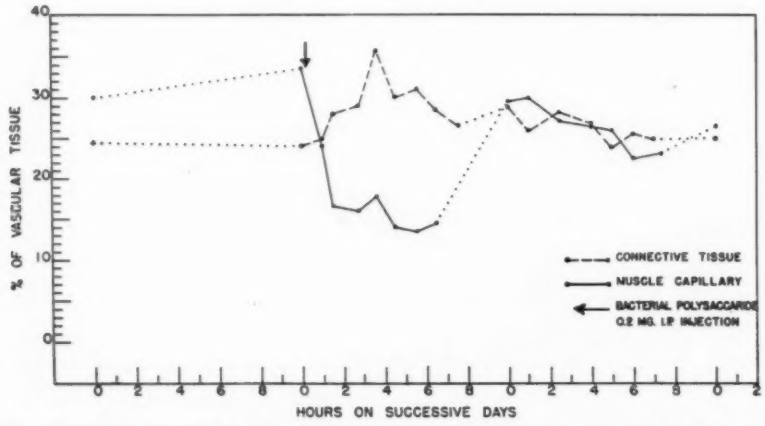

FIGURE 2.—Effect of bacterial polysaccharide on vascular level of capillaries of striated muscle.

and growth was resumed. There was no instance of complete and permanent destruction of any tumor under the conditions of these experiments. The greatest decrease in vascular levels was associated with the higher dose, 0.2 mg. The tumors used, dose of bacterial polysaccharide, quantitative changes in vascular level, occurrence of hemorrhage and necrosis, and mortality are shown in table 3.

An example of a maximum effect is given in detail. Figure 3, *A*, shows the vascular supply of a sarcoma 27 days after transplantation. At this time the tumor had a vascular level of 55 percent (fig. 4). One hour after injection of the polysaccharide, the tumor circulation had slowed considerably, and there was an apparent increase in circulating leukocytes. At the end of 2 hours, many tumor capillaries showed stasis, and the vascular level had dropped to 44 percent. This increase in stasis and disappearance of functional vessels proceeded rapidly, and by the end of 3 hours the vascular level had dropped to 24 percent. Numerous petechial hemorrhages (fig. 3, *B*) developed suddenly at the end of 3 hours and 45 minutes, during which time the tumor was held under continuous observation. Fluid and red blood cells surrounded the tumor. Because of stasis and occlusion of vessels, the functional vascular level dropped to zero at the end of 4 hours (fig. 4). The complete absence of functional vessels continued over the next 3 or 4 days. The tumor appeared to become completely necrotic, with ulceration at the base of the skin. Although the tumor was apparently completely necrotic by the fifth day after injection of the polysaccharide, new capillary sprouts became visible about the periphery, and tumor growth was resumed. During the next few days growth continued at a rapid rate, and the vascular

TABLE 3.—*Effect of bacterial polysaccharide on vascular levels in transplanted sarcomas* [1]

Experiment No.	Tumor	Strain of mice	Type of chamber	Dose	Vascular level									Hemorrhage	Partial necrosis	Tumor regression
					Starting hour 0	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	24 hours	48 hours			
				Mg.												
1	S37	C	Double layer.	0.1	42				26	20			27	+	+	−
2	S37	C	...do	.1	35			22		25		28		−	−	−
3	S37	C	...do	.1	48							41		+	+	−
4	S37	C	...do	.2	55					47			49	+	+	−
5	Earle's J strain.	C3H	...do	.2	51			41				0		−	+	−
6	...do	C3H	...do	.02	50					36			36	+	+	−
7	Earle's L strain.	C3H	...do	.02	53			52				42		+	+	−
8	...do	C3H	Single layer.	.2	52	38	37	42	26	33	31	6		+	+	−
9	Earle's D strain.	C3H	...do	.2	44	37	28	28	25			25	0	+	+	−
10	...do	C3H	...do	.2	57	58		24	0				0	+	+	−
11	Earle's H strain.	C3H	...do	.2	45	38	31		9			32		−	−	−

[1] Recovery in all experiments.

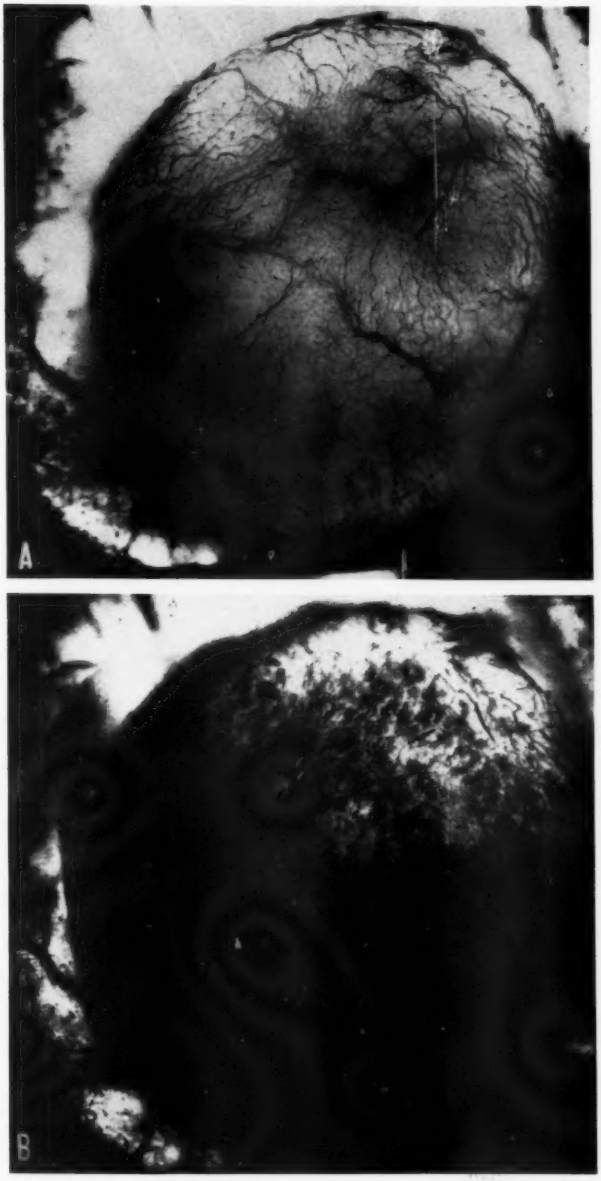

FIGURE 3.—*A*, Vascular bed of transplanted sarcoma prior to injection of bacterial polysaccharide. × 25. (Reduced one-half.) *B*, Hemorrhage in sarcoma approximately 4 hours after injection of bacterial polysaccharide. × 25. (Reduced one-half.)

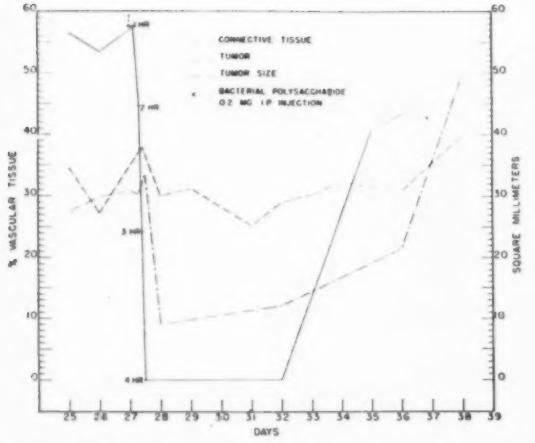

FIGURE 4.—Vascular reaction, in a transplanted sarcoma, to bacterial polysaccharide (single-layer chamber).

level in the growing area attained a height of 44 percent. Figure 5 shows the rich vascular supply that developed around the area of central necrosis. In addition to the evidence of edema in subcutaneous connective tissue previously referred to, it was observed that progressive development of vascular stasis resulted in edema in tumor tissue. In experiments in which evidence of edema was observed (table 3, experiments 1 to 7), the tumor was confined between two mica disks so that moderate external or internal pressure was sufficient to cause a significant drop in vascular level within the tumor. The result of injection of bacterial polysaccharide into a mouse carrying a chamber of this relatively rigid design is described as typical of the reaction of the tumor in this type of chamber. Figure 6, *A*, shows the tumor vascular supply prior to injection of 0.2 mg. of bacterial polysaccharide, and figure 6, *B*, the complete disappearance of functional vessels, as seen the following day. The increase in size of the tumor, as observed in figure 6, *B*, was probably due to edema resulting from progressive pressure damage to the capillaries. On the second day after injection of the polysaccharide, the entire tumor, as far as could be seen, became necrotic. Despite the apparent complete ne-

FIGURE 5.—Regrowth of tumor vascular bed around the necrotic area resulting from injection of bacterial polysaccharide. × 25. (Reduced one-third.)

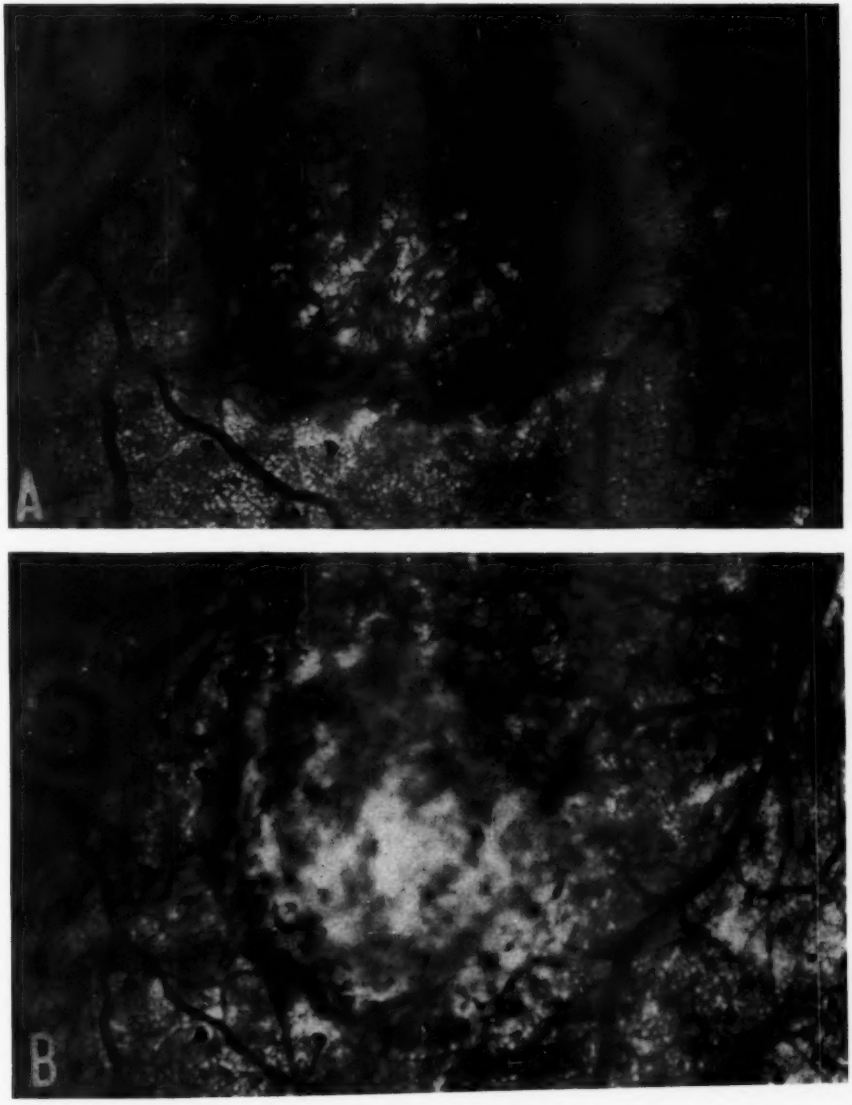

FIGURE 6.—*A*, Vascular bed of transplanted sarcoma prior to injection of bacterial polysaccharide. × 25. *B*, Vascular occlusion following injection of bacterial polysaccharide (double-layer chamber). × 25.

crosis of the tumor, it became evident 4 days after the injection that new capillary sprouts were arising about the tumor in the same manner as after the original transplantation. Coincident with the appearance of these new capillaries, increase in mass of the tumor became evident about the margin of the necrotic center. The quantitative data are given in figure 7. This experiment indicates that occlusion of the tumor vessels could be the result of internal pressure from either the accumulation of intercellular fluid, or the swelling of the tumor cells, or both of these factors. The subsequent events, hemorrhage and necrosis, would follow as outlined previously.

TRANSPLANTED MAMMARY-GLAND CARCINOMAS

Adequate experimental studies of the effect of the bacterial polysaccharide on transplanted mammary-gland carcinomas have not yet been carried out. Results of the two experiments performed,

however, were similar to those found when sarcomas were used, in that progressive slowing of the peripheral circulation was followed by vascular stasis and occlusion in the tumor. In the experiment shown in figure 8, it was possible to make

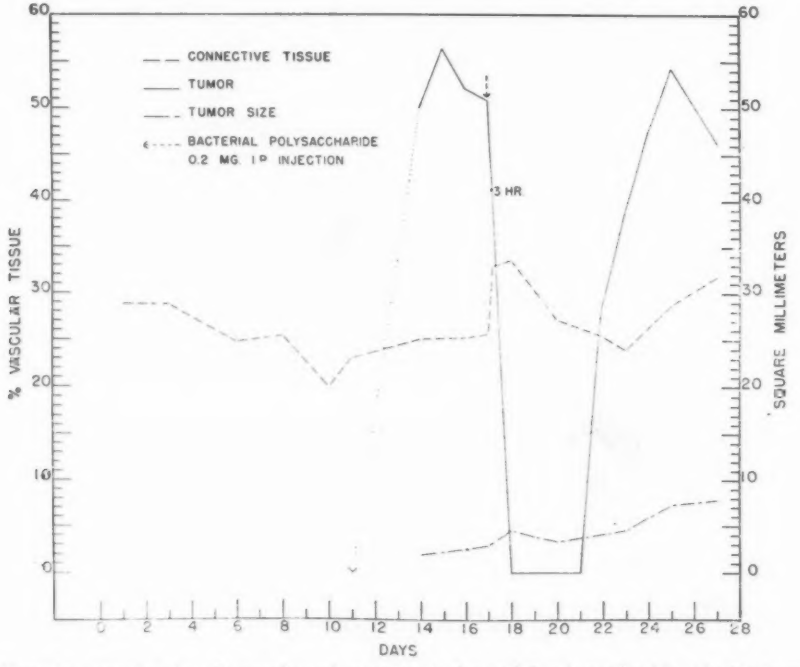

FIGURE 7.—Vascular reaction in a transplanted sarcoma to bacterial polysaccharide (double-layer chamber).

quantitative measurements of the capillary bed in both the tumor and the surrounding striated muscle, following injection of 0.02 mg. of the polysaccharide. It is apparent from this graph that vascular stasis and occlusion in the normal tissue were not dependent upon prior hemorrhage and necrosis in the tumor.

DISCUSSION

These experiments in transparent-chamber preparations have shown that following injection of this bacterial polysaccharide, hemorrhage and necrosis occur in the transplanted tumors within the transparent chamber after a preceding period of progressive slowing of the peripheral circulation followed by vascular stasis and occlusion of the capillaries both within the tumor and in normal striated muscle.

These experiments have also shown that the vascular stasis and occlusion produced by injections of this agent were not limited to tumor tissues, but that capillaries of striated muscle showed similar effects, followed by a cessation of the intermittent muscular contraction. Evidence is presented that vascular stasis resulted in tissue edema in both normal and tumor tissues. It can be assumed from the evidence of vascular stasis and tissue edema that capillaries of both normal and tumor tissues became increasingly permeable, and fluid loss occurred into the intercellular spaces (Krogh (*14*)).

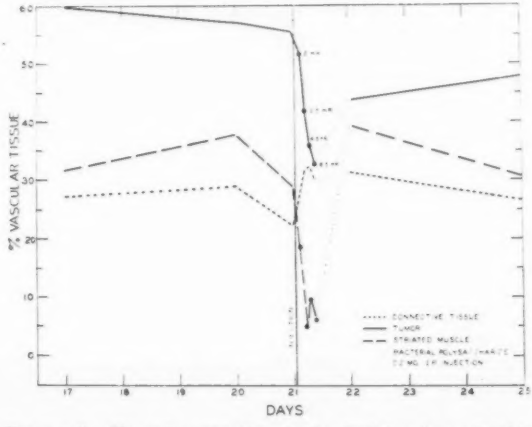

FIGURE 8.—Vascular reaction to bacterial polysaccharide in a transplanted mammary-gland carcinoma.

The opinion of many workers that the bacterial products are highly selective for tumor tissue was based on criteria of gross or microscopic evidence of hemorrhage or of cellular changes as seen in fixed and stained tissue sections. Apitz (*15*) stated that the striking results obtained in the tumor were confined characteristically to the tumor and were not part of a general vascular reaction to the administration of a toxic substance. Similar results were obtained by Shwartzman (*2*) in a study of tissue sections following administration of bacterial polysaccharide, and by Lippincott (cited by Shear (*10, p. 465*)). Zahl and Bjerknes (*16*), however, using endotoxin of gram-negative bacteria (*Shigella paradysenteriae*), were able to produce hemorrhage not only in transplanted tumors but also in decidua-placental tissues. Recently Diller and Shear (*17*) presented cytologic evidence of damage to cells of intestinal epithelium as well as of tumors of mice following injections of the bacterial polysaccharide.

Inferences about the mechanism of action of the bacterial products in producing hemorrhage and necrosis in tumors have been based on the microscopic examination of tissue sections. Apitz (*15*) concluded from such studies that the walls of capillaries in the tumor were damaged, that this led to dilatation and rupture of the walls of the blood vessels, and that the resultant bleeding obliterated the capillary wall. Andervont (*5*) also concluded that the vessels become dilated and rupture, causing a diffuse hemorrhage throughout the tumor. Both of these workers noted an edematous appearance of some of the tumor cells prior to the hemorrhage, inferring from this that the bacterial products may have some direct effect upon the tumor cells. Gerber and Bernheim (*18*) from examination of tissue sections found no evidence of vascular alterations and concluded that the effect seems to be directly upon the tumor cells.

From the experiments presented here, it appears that the hemorrhage and necrosis within the tumor are probably manifestations of capillary damage due to tissue anoxia resulting from circulatory slowing throughout the host. This circulatory slowing is primarily a host reaction to which tumor tissue responds by hemorrhage and necrosis. The reasons for the differences between the reaction to circulatory stasis of capillaries in the striated-muscle layer and in sarcoma must be sought in comparative studies of such factors as capillary fragility, pressure, permeability, and reaction to anoxia. That tissue anoxia could lead to both hemorrhage and cellular degenerative changes is borne out by the results of other workers. Markee (*19*), for example, described anemia of endometrial transplants in response to injection of estrin, hemorrhage resulting 24 hours later when circulation was resumed. Windle (*20*) showed that fetal guinea pigs, resuscitated after being subjected to anoxia by clamping the maternal uterine vessels for varying periods, exhibited multiple capillary hemorrhages, brain edema, and neurocytologic changes, when killed one-half hour to 21 days later.

Shear (*10*) discussed the question whether the bacterial products produce hemorrhage in tumors as a result of severe shock in the host. He pointed out, however, that—

> a distinction should be made between the production of severe shock in the host resulting in hemorrhage in tumors, and the production of severe hemorrhage in tumors resulting in shock in the host. In both instances hemorrhage and shock are obtained, but the sequence of events is reversed.

In the development of both tourniquet shock and the reaction to the intraperitoneal injection of bacterial polysaccharide, there is a drop in the vascular level in the capillary bed of the striated-muscle layer of the skin (*21*). This seems referable in both cases to a decrease in blood volume. In tourniquet shock fluid is lost at the site of injury, and there is no evidence of an initial systemic increase in capillary permeability. After bacterial polysaccharide, however, evidence was obtained of a systemic increase in capillary permeability as manifested by edema of the subcutaneous tissues. After either procedure, there resulted decreased capillary blood flow, leading to tissue anoxia and consequent damage.

These results suggest that the mechanism of action of this bacterial polysaccharide is one example of a common host reaction to a variety of agents which similarly decrease the host circulatory rate, secondarily affecting the tumor circulation with resulting irreversible anoxic damage. That this may be the case is borne out by recent preliminary experiments [6] on the mechanism of damage to tumors following release of tourniquets applied to the hind legs of mice, and of the action of podophyllin on tumors. In both of these instances, hemoconcentration, sluggish circulatory rate, and capillary stasis occurred in normal and in tumor tissue with varying degrees of hemorrhage and necrosis in the tumors.

These experiments serve to emphasize the need for critical studies of the mechanism of action of agents which affect tumors, not only by the relatively static methods of classic pathology and cytology, which can usually reveal only end results, but also by some of the more dynamic methods of pathologic physiology, which seem to be of particular significance in differentiating between primary and secondary effects of an agent.

In instances where the tumor was severely damaged but the host survived, the peripheral surviving tumor cells were capable of arousing renewed capillary proliferation and thus restored their nu-

[6] ALGIRE, G. H.: Unpublished experiments.

tritive supply. It seems, therefore, that an important field for further biologic and chemical study is the factors concerned in the development of the tumor blood supply through capillary proliferation from the host.

SUMMARY

Photographs and daily quantitative measurements were made of the vascular bed in the normal (striated muscle) and neoplastic (transplanted sarcoma) tissue included within a transparent chamber introduced into a dorsal skin flap in the mouse, prior to and following intraperitoneal injection of a polysaccharide fraction from *Serratia marcescens* (*Bacillus prodigiosus*).

The following results were obtained: Progressive decrease in the rate of blood flow and percentage of functional vessels in the capillary circulation to the panniculus carnosus layer of striated muscle and to transplanted sarcomas. This quantitative decrease in functional capillaries was accompanied by stasis and occlusion. Capillary dilatation was not observed in either striated muscle or tumor tissue.

In the capillaries of the striated-muscle layer, the vascular occlusion reached a maximum in from 3 to 4 hours; and in animals that survived, recovery of capillary flow occurred within 18 hours. Neither hemorrhage nor necrosis was observed in these normal tissues, but muscle contraction ceased as capillary circulation decreased.

Evidence was obtained that the vascular occlusion and stasis in both normal and neoplastic tissues were associated with edema, which suggested increased capillary permeability.

The development of vascular occlusion in transplanted sarcomas was followed after varying periods by the sudden appearance of petechial hemorrhages throughout the tumor. In spite of the apparently complete necrosis which developed, some peripheral tumor cells survived. This surviving tissue retained its capacity to elicit a vigorous vascular supply from the host, and growth of the tumor was resumed in every instance.

No evidence of a primary action of this agent on tumor cells was obtained.

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PULMONARY-TUMOR INDUCTION BY TRANSPLACENTAL EXPOSURE TO URETHANE[1,2]

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INTRODUCTION

With but one exception, the literature appears to be devoid of reports pertaining to intra-uterine exposure of embryonic tissue to the action of carcinogenic material. Law (*1*) subjected embryonic mice, Caracul × P, F_1 hybrids, in utero (14-day stage) to 1,2,5,6-dibenzanthracene. Eighty-three percent of the surviving young exhibited multiple primary pulmonary tumors when autopsied at an average age of 6⅓ months.

Following the original report (*2*) that repeated narcotization with ethyl carbamate (urethane) was followed by striking increases in the incidence and multiplicity of pulmonary tumors in strain A mice, it was observed (*3, 4*) that carcinogenesis was started in the absence of clinical narcosis and that the reaction of pulmonary tissue to urethane appeared to be independent of the route of administration of a fixed total dose. Subsequently, the writer[3] has observed significant increases in the incidence and the multiplicity of lung tumors in strain A mice following a single intraperitoneal injection of a subnarcotizing dose of urethane (0.25 mg. per gram mouse). Although urethane has not been examined at the minute dosage levels found effective for the more potent of the carcinogenic hydrocarbons, available data suggest that it is of a lower order of carcinogenicity. In extending the examination of the urethane-pulmonary tumor phenomenon, the results reported herein are from a series of experiments which were performed in demonstrating that urethane under certain conditions introduces a carcinogenic process in fetal lung tissue.

EXPERIMENTAL PROCEDURE AND RESULTS

SERIES A

Pulmonary tissue of strain A adult mice is more susceptible to the carcinogenicity of urethane than is that of any other strain examined.[4] On the supposition that this greater sensitivity might be encountered in embryonic tissue, this strain was used in all experiments described herein. By way of exploration, a small number of pregnant mice from the breeding colony were narcotized late in term by intraperitoneal injection of 25 mg. of urethane. At 6 months of age, the 51 surviving offspring were killed by cervical dislocation, and the lungs were examined for pulmonary tumors. Forty mice exhibited 1 to 19 lung tumors each. Inspection of the animal records showed that tumors were present in all mice that had been born during the 24-hour period after injection of their respective mothers. Tumor incidences were 85 and 63 percent, respectively, in offspring born during the second and third 24-hour post-injection periods. Similarly, the data in table 1 indicate that an inverse relationship exists between the number of tumors and the injection-parturition interval.

The available data for the relatively small numbers of mice were subjected to limited statistical analyses. The tumor incidence in each experimental group differed significantly from that in control mice; $P<0.001$ in each instance. Differences in incidence among the experimental groups, however, approached significance only when 1-day and 3-day mice were compared, $P=0.01$. The average number of tumors in each group of offspring was significantly greater than in control mice, $P<0.001$ for each mean difference. Although of a lower order, tests also showed significant differences between the means of the experimental groups: One-day versus two-day mice, $t=2.42$; $P=0.02$; 1-day versus 3-day mice, $t=5.24$, $P<0.01$; 2-day versus 3-day mice, $t=2.56$, $P=0.01$.

TABLE 1.—*Transplacental carcinogenesis, preliminary tests*

Series	Number of mothers	Injected after plug	Litters		Offspring		Average tumors per mouse
			Born	Interval after last injection	Living 6 months	With lung tumors	
		Days	*Number*	*Days*	*Number*	*Percent*	*Number*
A	13		4	1	14	100	7.9
			4	2	13	85	3.1
			4	3	24	63	1.1
B	12	2, 9, 16	2	4	(1)		
C	12	4, 11, 18	1	1	3	100	9.7
			1	2	2	50	.5
			3	1	(1)		
D	24	6, 12, 18	7	1	12	100	7.4
			2	2	4	75	.75
			9		(1)		
E	38	8, 16	12	3	65	58	.9
			9	4	38	60	1.1
			6		(1)		
Controls					158	17	[2] 19

[1] Litters not recorded.
[2] 3 of 27 tumor mice exhibited 2 tumors each at 6 months of age.

In the foregoing experiment, the pulmonary-tumor data demonstrated that some action had been started in fetal-lung tissue, particularly in those mice whose mothers had been injected late in term, which resulted later in increases in the incidence and multiplicity of tumors. Conse-

[1] Received for publication August 28, 1947.
[2] Presented in part at the meetings of the American Association for Cancer Research, Chicago, May 16–17, 1947.
[3] Unpublished data.
[4] Unpublished data.

quently, it seemed that several narcotizing injections of the gravid mouse might result in an enhanced effect. Accordingly, several series of females were bred and then injected intraperitoneally several times during the resulting pregnancies. The relationships of the injections to the stages of embryonic development are noted in the following experiments.

Female mice 8 to 10 weeks of age were mated with males of the same age. The females were inspected daily, and vaginal plugs when present were noted. In series B, C, D, and E, two or more injections were made at arbitrary intervals after appearance of the vaginal plug. Each injection consisted of 25 mg. urethane in 0.25 ml. distilled water. After parturition and weaning, the offspring were maintained on the National Cancer Institute stock diet No. 77 (*5*) (in pellet form) and water. At 6 months of age, the offspring were killed by cervical dislocation, the lungs were removed, and all surfaces of each lobe were examined under the dissecting microscope (×7 magnification). Most of the tumors observed were as large as or larger than the spontaneous lung tumors seen in older mice of this strain. The tumors were readily identified by their pearly appearance, their relative nonfriability, and their tendency toward subpleural elevation and occasional pedunculation. The smaller, grossly questionable nodules were routinely fixed and stained for microscopic verification. Random samples of grossly diagnosed tumors were invariably confirmed when examined microscopically.[5]

Series B

Twelve female mice were injected intraperitoneally with 25 mg. urethane on the second and ninth days after the vaginal plug was noted. By the fourteenth day it was obvious that pregnancy had failed to develop in 10 of these mice, perhaps because of interference with uterine implantation. The other 2 mice, injected a third time on the sixteenth day, cast stillborn litters on the twentieth day.

Series C

Twelve female mice were injected on the fourth, eleventh, and eighteenth post-plug days. In 7 mice pregnancy was not completed. The other 5 mice delivered litters at term, 2 of which were reared. One litter, born during the 24-hour period following the third injection of the mother, consisted of 3 mice which at 6 months of age exhibited 7, 8, and 14 lung tumors, respectively. In the other litter, in which the interval between the final injection and parturition was 2 days, 1 of 2 mice exhibited a single lung tumor at 6 months of age.

[5] Dr. Thelma B. Dunn, of the National Cancer Institute, made the histologic examination of the tissues.

Series D

Twenty-four female mice were injected on the sixth, twelfth, and eighteenth post-plug days. Normal pregnancy developed in 18 mice, 9 of which reared their litters. Seven of the litters were born in the 24-hour period following the final injection of the mother. Twelve surviving offspring were examined at 6 months of age; all had lung tumors, 4 to 14 per mouse, average 7.3. The other 2 litters, born on the second day, following the final injection, provided 4 mice for autopsy at 6 months of age; 3 had 1 tumor each, 1 mouse had none. Control mice of the same age on the average exhibited lung tumor in every fifth or sixth mouse.

Series E

Thirty-eight female mice were injected in the same manner with urethane on the eighth and sixteenth post-plug days. Twenty-seven litters were carried to term, of which 21, consisting of 103 mice, were reared. These offspring exhibited a pulmonary tumor incidence of 59 percent and an average of 1.0 tumor. These values, although definitely lower than those for the 1-day mice in series A, C, and D, demonstrate some transplacental carcinogenicity when compared with data for control mice.

As a result of the preliminary experiments, series A through E, and in spite of a lack of uniformity in the number of injections and their relationship to embryonic development, the data in table 1 suggest that fetal-lung tissue is most susceptible to the transplacental carcinogenic action of urethane during the 24 hours preceding parturition. These data are extended in the following experiments.

Series F

Virgin females, 8 to 10 weeks of age, were mated with young, vigorous males. The females were examined at least once daily for the presence of vaginal plugs. As plugs were found, the mice were assigned to one or another of 5 groups. The mice in the several groups were injected intraperitoneally once only with 25 mg. urethane on the fourteenth, fifteenth, sixteenth, seventeenth, or eighteenth post-plug days. In order to facilitate analysis of the resulting experimental material, the offspring were regrouped after parturition. Assignment of offspring to groups was on the basis of the interval between injection of the mother and delivery of the young, i. e., those mice born within the first 24-hour period following injection of the mother were assigned to the 1-day group (table 2), those delivered during the second 24-hour post-injection period to the 2-day group, etc. In the event of a missed plug, the mouse was injected late in term, and the offspring were assigned to the appropriate group, post partum, for purposes of

analysis. The young mice were reared and at 6 months of age were killed and examined for pulmonary tumors. Table 2 shows that the incidence of lung tumors was 100 percent in that group whose mothers had been injected during the 24-hour period preceding parturition. An average of 8.9 tumors per mouse was observed. In the mice born after intervals of 2, 3, 4, or 5 days, the tumor incidences, 76, 61, 62, and 83 percent, respectively, which appear to be more or less independent of the injection-parturition interval, were less than that for the 1-day group, but considerably higher than the usual incidence of 15 to 20 percent in controls of the same age. Even more striking is the observation that the multiplicity of tumors in the 1-day group is fully 5 times greater on the average than in mice born after longer injection-parturition intervals. If only mice exhibiting tumors are considered, the ratio of multiplicity in the 1-day group to that of any other group is about fourfold. Groups 2, 3, 4, and 5 exhibited tumor multiplicities which diminished but slightly as the interval increased from 2 to 3 to 4 to 5 days. Essentially identical results were obtained in series G. Thirty-nine of the intraperitoneally injected mothers, series F, were killed and examined 6 months after parturition. All mice but 1, in which no tumor was found, had developed multiple lung tumors, 3 to 38 per mouse (average, 17.7). Variation from mouse to mouse in response to the agent is given in table 3.

Series G

In the experimental work just described, all injections of gravid mice were made intraperitoneally. It was recognized that this technique is subject to the criticism that intraperitoneal injection of a mouse late in term might be intrauterine in some instances or perhaps occasionally within the amniotic sac. The latter possibility is remote as well as of little consequence, for injection of an appreciable quantity of urethane directly into the amniotic fluid would in all probability result in the death of that embryo, and its resultant exclusion from the data on 6-month survivors. In order to invalidate potential criticism of the foregoing data for these or other reasons, a final experiment was performed in which all pregnant mice were injected intravenously. All other experimental conditions duplicated those for the preceding experiment. Each injection was made into a tail vein. With experience, it was found that intravenous injection of 0.25 ml. of a 10-percent solution (25 mg.) of urethane required that administration be performed uniformly during a period of not less than 60 seconds; otherwise, when the injection proceeded more rapidly, many animals died immediately because of what appeared to be respiratory failure. The onset of anesthesia, almost instantaneous, was much more rapid than when the intraperitoneal route was used; likewise, because of more rapid metabolism and excretion of the drug, narcosis was more transient. In the event that intravenous injection was not successful, owing to the repeated failure of insertion of the needle into a vein, the mouse was injected subcutaneously. Litters of mice so injected were excluded from the tabulated data. However, exam-

TABLE 2.—*Pulmonary-tumor induction in mice*

Series	Route of injection of mothers	Injection-parturition interval	Offspring: Number	Offspring: With tumors	Offspring: Average tumors per mouse	Mothers [1]: Number	Mothers [1]: With tumors	Mothers [1]: Average tumors per mouse
		Days		*Percent*	*Number*		*Percent*	*Number*
F	Intraperitoneal	1	40	100	8.9	6	83	17.4
		2	29	76	1.8	8	100	18.0
		3	111	61	1.2	9	100	15.9
		4	58	65	1.3	7	100	18.6
		5	42	83	1.5	9	100	20.8
Total or average.						39		17.7
G	Intravenous.	1	73	100	10.0	22	100	24.8
		2	57	82	1.7	18	100	24.0
		3	82	73	1.5	25	100	22.1
		4	71	76	1.6	16	100	21.8
		5	84	73	1.4	23	100	26.9
Total or average.						104		24.0

[1] Mothers each received 25 mg. urethane, injected as indicated.

TABLE 3.—*Pulmonary tumors in strain A mice 6 months after single injection of urethane*

Route [1]	Mice showing given number of tumors per individual: 0	1	2	3	4	5	6	7	8	9	10	11	12
I. P.	1	....	....	1	1	....	....	....	1	1	1	3	....
I. V.	....	1	2	1	....	2	1	....	1	1	....	2	1

Route [1]	Mice showing given number of tumors per individual: 13	14	15	16	17	18	19	20	21	Number of mice	Average tumors per mouse
I. P.	2	3	2	1	2	2	2	2	3	.....	.....
I. V.	1	....	3	3	4	3	2	1	8	.....	.....

Route [1]	Mice showing given number of tumors per individual: 22	23	24	25	26	27	28	29	30	31	32	33	34
I. P.	2	1	1	1	1	....	2	1	....	....	....	....	1
I. V.	4	5	2	4	9	2	10	3	4	3	3	2	5

Route [1]	Mice showing given number of tumors per individual: 35	36	37	38	39	40	41	....	48	Number of mice	Average tumors per mouse
I. P.	....	....	....	1	....	....	....	....	....	39	17.7
I. V.	4	1	....	1	3	....	1	....	1	104	24.0

[1] I. P., intraperitoneal; I. V., intravenous.

ination of 6-month-old offspring of subcutaneously injected mothers showed essentially the same lung-tumor incidence and multiplicity, injection-parturition intervals being the same, as was observed in offspring of intraperitoneally or intravenously injected mothers. The offspring of intravenously injected animals were killed at 6 months of age, examined for lung tumors, and the data tabulated according to the length in days of the injection-parturition interval. The data in table 2 coincide closely with those from single intraperitoneal injections (series F). Tumors were found in the lungs of all mice born during the 24-hour period following injection of the mothers, average 10.0. Those offspring born after 2-, 3-, 4-, or 5-day intervals had tumor incidences of 82, 73, 76, and 73 percent, respectively. On the basis of all animals examined, the average number of tumors in each of these groups was about one-sixth of that in the 1-day group, or about one-fifth when the average was computed on the basis of tumor animals only.

Each surviving mother in series G was killed at the same time as her offspring and examined for lung tumors. Adenomas were found in all mice, 1 to 48 per mouse, average 24.0. Variability of response to the agent is shown in table 3.

DISCUSSION

The data of the several exploratory experiments, series A through E (table 1) representing small groups of mice are too limited to warrant more than brief attention. The values for incidence and multiplicity of tumors in the 6-month-old offspring in the several series indicate that urethane, administered intraperitoneally to the pregnant mouse, effected transplacental carcinogenesis in the fetal lungs. The data suggest that the magnitude of the effect was greater in the offspring delivered of mothers which had been injected during the terminal 24 hours of gestation than in those from mothers injected earlier. In series A, all mice born during the 24-hour post-injection period exhibited lung tumors at 6 months of age, with an average of 7.9 tumors per mouse. Following injection-parturition intervals of 2 and 3 days, the offspring exhibited lower tumor incidences and fewer average tumors per mouse. Essentially the same relationship of tumor response to the injection-parturition interval existed in series C and D. In series E, in which all offspring were born either 3 or 4 days after the second injection of the respective mothers, the incidence and multiplicity of tumors were of the same order as in the 3-day-interval mice in series A. These values, although definitely less than those for the 1-day-interval mice in series A, C, and D, nevertheless differ significantly from data for control mice (table 1). The evidence obtained from the exploratory experiments is extended and substantiated in series F and G.

All offspring in series F born within 24 hours after injection of the mother had pulmonary tumors when examined at 6 months of age; an average of 8.9 tumors per mouse was noted. In contrast, significantly lesser tumor incidences and strikingly fewer numbers of lung tumors were found in mice born after intervals of more than 24 hours, i. e., 2, 3, 4, or 5 days after injection of their mothers. Application of the χ^2 formula showed that the tumor incidence in the 2-, 3-, 4-, or 5-day offspring was in each instance significantly less than that of 100 percent in the 1-day-interval offspring; $\chi^2=11$, 22, 17, and 7, respectively; $P<0.001$, <0.001, <0.001, and <0.01. On the other hand, the tumor incidence in the 2-day mice did not differ significantly from that in the 3-day mice, or that in the 3-day mice from the 4-day, or the 4-day from the 5-day mice. Nevertheless, comparison of the data for mice born after injection-parturition intervals of more than 1 day with that for control mice (table 1) showed highly significant differences; $\chi^2=44$, 56, 47, and 69, respectively, $P<0.001$ in each case.

The definitely greater susceptibility of fetal pulmonary tissue to urethane during the final day of embryonic life is further demonstrated when one examines the data for the average number of lung tumors in the 1-day mice and the averages in those born after longer intervals. In the animals born during the 24-hour period following injection of their mothers, an average of 8.9 tumors per mouse was noted. This mean differs from the means in the 2-, 3-, 4-, and 5-day mice by 7.1, 7.7, 7.6, and 7.4. The smallest of these differences, that between the 1-day and the 2-day mice, was tested and found to be statistically significant ($t=8.10$; $P<0.001$). The other differences were of the same order of significance. Examination of the means in the 2-, 3-, 4-, and 5-day mice shows, however, that any one mean does not differ significantly from that of any other of these four groups. These means differ from that in the control mice by 1.61, 1.01, 1.11, and 1.31, respectively. Analyses of these differences show the same order of significance in each case. For example, the mean of 1.3 in the 4-day mice differs from the mean of 0.19 in control mice by 1.11 ($t=9.68$; $P<0.001$).

In series G, wherein the offspring were born of intravenously injected mothers, the response to the agent was essentially the same as in comparable groups in the intraperitoneally injected series. In the 1-day mice, the 100-percent incidence of tumors was significantly greater than the incidences of 82, 73, 76, and 73 in the 2-, 3-, 4-, and 5-day mice. Respective χ^2 values of 17, 22, 20, and 23 were observed; $P<0.001$ in each instance. Comparison of incidences in the 2-, 3-, 4-, and 5-day groups, one

with another, failed to demonstrate any significant differences. The incidence in each of the last four groups, however, was found to be highly significant when compared with the incidence of 17 percent in the controls (χ^2=79, 73, 74, and 73; $P<0.001$ in each case).

The mean number of tumors in the group born within the 24-hour period following intravenous injection of their mothers differed from the means in the 2-, 3-, 4-, and 5-day groups by 8.3, 8.5, 8.4, and 8.6, respectively. The smallest of these differences, that between the 1-day and the 2-day mice, was statistically significant ($t=9.661$; $P<0.001$). The differences between the 1-day and the 3-, 4-, and 5-day means were of the same order of significance. However, comparison of the means of the groups born 2, 3, 4, and 5 days after maternal injection, one with another, failed to demonstrate any significant differences. Again, the difference between the tumor mean in each of the 2-, 3-, 4-, and 5-day groups and that in the controls was found to be significant. For example, the smallest of the several differences, that between the means of the 5-day group and the controls, was 1.21 ($t=13.1$; $P<0.001$).

From the available data, it must be concluded that the route of administration of urethane to pregnant mice, intraperitoneally and intravenously in series F and G, respectively, is not a factor in the degree of carcinogenesis attained in the lungs of the offspring. The difference of 1.1 between the means of the 1-day group in series F and the 1-day group in series G is not significant ($t=0.97$; $P=0.3$). Similar probabilities result on analysis of the means of comparable groups born after longer intervals in the two series.

The data pertaining to lung-tumor elicitation in the maternal mice in series F and G merit brief attention. Variations in the injection-parturition interval do not appear to be reflected in significant differences in the mean numbers of lung tumors observed 6 months later within the groups of either series. However, the mean number of lung tumors of all intraperitoneally injected mothers (table 2) differs significantly from that of all intravenously injected mothers ($t=3.74$; $P<0.001$). The more rapid development of narcosis in mice injected intravenously with urethane than when the same dose is administered intraperitoneally is obviously the result of a more rapid and higher peak concentration in the blood stream; also, the greater tumor elicitation in the intravenously injected mothers is perhaps a manifestation of a relatively higher concentration of urethane.

The foregoing data and the analyses thereof clearly demonstrate that urethane administered late in term to the gravid mouse results in striking increases in the incidence and multiplicity of pulmonary tumors in the surviving offspring. The data also show that the fetal lung is most susceptible to the agent during the last 24 hours of intrauterine life. Whereas the experimental results are clear-cut, their explanation is less certain. Since the experimental results in series F and G, in which the offspring were born of intraperitoneally and intravenously injected mothers, respectively, are essentially identical, the following discussion is applicable to either series.

On the assumption that fetal-lung tissue in strain A mice was susceptible to tumor elicitation with urethane, it should have been and was anticipated that a transplacental effect would be attained in some degree. Barcroft and co-workers (*6*) observed evidence of anesthesia in fetal lambs following maternal narcotization with urethane. Similar fetal anesthesia in other species was noted by others (*7, 8, 9, 10*) with other anesthetics. Urethane is readily soluble in both aqueous and lipid media. After administration to the gravid mouse, this low-molecular-weight compound would be expected to enter the fetal circulation mainly by way of the placenta and, perhaps in small amounts, by extraplacental diffusion from maternal tissues into amniotic fluid.

That urethane concentrations in the fetal circulation can reach a level sufficient to bring about events leading to later appearance of lung tumors significantly greater in number than in control animals, is established by the data in tables 1 and 2. These data demonstrate that offspring exposed to the agent during the final 24 hours in utero subsequently develop fully five times as many lung tumors as do mice whose mothers were injected 2 to 5 days ante partum. In the absence of supporting information in this species and strain, an explanation of the striking increase in response to late-in-term administration of the agent can be only conjectural. Either the 1-day offspring are more susceptible to the agent or their lungs are exposed to more of it. With regard to the former alternative, an increase in susceptibility in pulmonary tissue during the last 24 hours of gestation, which would result in a fivefold increase in the number of tumors, is improbable. To attribute the increase in tumors to a corresponding increase in the number of cells in fetal lungs is not warranted. Explanation of the larger number of tumors in the 1-day offspring on the basis of an increased quantity of urethane or a metabolite which comes into intimate contact with fetal pulmonary tissue is given support by information concerning other species.

It is known that degeneration associated with increased permeability occurs in the placenta prior to and during the onset of labor. Such a change in the maternal-fetal relationship, if it takes place in the mouse, might account for the postulated increase in the level of urethane in the fetal circulation. It might be that the known permeability of

the placenta to maternal estrogens increases near term and that resulting increases in hormone titer in the fetal circulation condition the fetal lung to greater susceptibility to the agent by some as yet unknown mechanism. Unpublished data tend to refute this possibility, as no significant sex difference has been observed in the tumor response of young adult strain A mice following single or repeated injections of urethane. With or without possible increases in placental permeability, hormonal modifications, and increases in competency or numbers of cells, the tumor response in the 1-day offspring might be the result of late-in-term modifications in fetal circulation or physiology, or both.

Circulation has been studied extensively in near- and full-term fetuses of many species of experimental and domesticated animals and in man by many investigators. Unfortunately, in so far as the writer is aware, similar studies have not been extended to the mouse. Analogies which may be drawn in an attempt to explain the results of the present experiment are, perforce, predicated upon the presumption that maternal-fetal relationships and fetal circulation in the mouse and in larger animals are essentially the same from anatomical and functional aspects. An extensive review of the literature dealing with fetal circulation is beyond the scope of this report. The interested reader is referred to the review and experimental work of Barclay, Franklin, and Prichard (*11*), the extensive works of Barcroft, Barron, Windle, and associates, Snyder and Rosenfeld, and others. In the genera and species studied in vivo the fetal circulation, roughly speaking, appears to be as follows: Oxygenated blood flows from the placenta by way of the umbilical veins to the fetal liver, to the posterior vena cava where it is joined by hepatic veins, portal inflow, and venous return from more caudal parts of the body. The posterior caval channel bifurcates as it enters the heart, and most of the blood, in contrast with the situation in the postnatal or adult heart, enters the left atrium and is pumped by the left ventricle into the ascending aorta. Here some blood enters the coronary arteries, a larger portion enters the arteries supplying the cephalad parts of the body, and the remainder traverses the aortic arch to the descending aorta from which it is distributed to the abdominal and caudally located arteries, and to the umbilical arteries, and back to the placenta. At the cardiac bifurcation the lesser portion of the posterior vena caval flow joins the entire anterior caval flow in the right atrium and is pumped by the right ventricle through the pulmonary valve to the pulmonary trunk. To this point, the anterior caval blood of the fetus follows the same course as that in the adult, but beyond this point the flow is different. In the postnatal or adult circulation, the blood in the pulmonary trunk enters the pulmonary arteries and returns to the left atrium by way of the pulmonary veins. In the fetus, the pulmonary arteries are largely bypassed, as the blood from the pulmonary trunk flows in large part through the ductus arteriosus and unites with the descending aorta. The smaller portion of the blood which does not enter the ductus arteriosus flows through the pulmonary arteries, the lung capillaries, the pulmonary veins, and thence to the left atrium. Since fetal lungs are not concerned with respiration per se, the blood entering the pulmonary arteries and capillaries serves only to supply the developing pulmonary tissue with anabolic requirements. The function of the ductus arteriosus in the fetal circulation, particularly that in the lamb, has been demonstrated in a series of papers by Barclay and co-workers. Pertinent radiographic evidence of the prenatal circulation and of the functioning ductus has been collected by this group (*11*). Their own work, together with that of other investigators which they review on rabbit, puppy, guinea pig, kid, and human fetuses, indicates that functional closure of the ductus arteriosus begins immediately after parturition and the beginning of concomitant dilatation of the lungs by air and is complete within a matter of minutes. Anatomical closure requires a period of days or weeks. Obviously, if fetal circulation, and the changes which it undergoes at or following birth, in the mouse is the same as or similar to that in larger genera, one is hard pressed to correlate the sharp increase in susceptibility of fetal mouse lungs to urethane during the last day of intra-uterine existence with corresponding increases in the flow of blood through the pulmonary arteries and capillaries of the fetus. However, it must not be overlooked that the radiographic techniques of Barclay and others, advanced and elucidative as they are in other species, are essentially qualitative. It might readily be postulated that as gestation approaches termination the fetus prepares itself for the sudden change from placental to pulmonary respiration. In order that the lungs be ready to take on their postnatal function of aeration of the blood, an increased flow in the pulmonary arteries and capillaries might be expected as parturition becomes imminent. A patent ductus arteriosus at the time of parturition, in the species studied by Barclay and others, does not necessarily preclude an increased volume of flow in the already functioning prenatal pulmonary arteries. An increase in pulmonary vascularity just prior to parturition is made more plausible by other evidence.

The late-in-term increase in susceptibility of the fetal lungs to the beginning of events that lead to a later appearance of lung tumors might be associated either directly or indirectly with the phenomenon of prenatal respiratory movements. Evidence of this fetal activity has been reviewed by

Windle (*12, 13*). The existence of prenatal rhythmical movements taking the form of shallow rapid breathing and sometimes of dyspneic gasping seems to be generally accepted by investigators in the field. Whether such movements are associated with aspiration of amniotic fluid is controversial. The evidence reviewed by Windle (*12*) and Whitehead, Windle, and Becker (*14*) suggests that aspiration of fluid, observed in varying degree by several investigators, may have been the result of experimental conditions. Windle and co-workers (*8*) observed aspiration of fluid into the trachea and bronchi but not into the alveoli of fetal guinea pigs in which the oxygen level in the blood had been lowered. These investigators appear to take the position that apnea is the rule in the physiologic embryo, and that respiratory movements, when observed experimentally, are the result of fetal anoxemia caused either by maternal anoxemia or by interference with maternal-fetal transport. On the other hand, Snyder and Rosenfeld (*10*), in experiments in which disturbances of physiologic conditions were minimal, observed anoxemic inhibition of respiratory movements. In rabbit, cat, and guinea-pig fetuses, they frequently observed respiratory movements, described as rhythmical excursions of the chest wall and diaphragm, through periods of several hours. The phenomenon appeared to be most frequently observed at full term. If one assumes that there are respiratory movements in the fetal mouse during the final 24 hours of intra-uterine environment, this phenomenon, in either of two ways, might increase the effective dose of urethane which contacts susceptible tissue.

Urethane, particularly when administered intravenously, markedly slows and sometimes completely inhibits maternal respiration. If the inhibition of respiration in the mother is reflected by an oxygen want in the late-in-term fetus, recourse to the position taken by the Windle group permits acceptance of the postulation that such anoxemia may bring about fetal respiratory movements when absent and stimulate them when they are present. Such movements might be expected to stimulate additional flow of blood through the pulmonary arteries even in the presence of a patent ductus arteriosus. If respiratory movements are accompanied by aspiration of amniotic fluid, such an occurrence could be a factor in increasing the quantity of the agent contacting susceptible pulmonary tissue.

The strikingly greater number of lung tumors encountered in mice whose mothers had been injected with urethane during the final 24 hours of gestation than in offspring born after long injection-parturition intervals, may be caused by one or more of the physical and physiologic factors discussed.

SUMMARY

Offspring of strain A mice injected late in term with urethane exhibited significant increases in incidence and multiplicity of pulmonary tumors when examined at 6 months of age. The degree of tumor elicitation appeared to be the same in offspring of mothers injected intraperitoneally or intravenously.

Offspring of mothers injected during the final 24 hours of gestation exhibited fully five times as many lung tumors as did mice whose mothers were injected 2 to 5 days ante partum.

Gravid mice that received a single intravenous injection of urethane developed 33 percent more lung tumors than did similar mice injected intraperitoneally.

Physical and physiologic conditions associated with intra-uterine environment are discussed in the light of their possible roles in the sharp increase in susceptibility of fetal mouse lungs to the agent during the last 24 hours of gestation.

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ENZYMATIC HYDROLYSIS OF HOMOLOGOUS AMINO ACID AMIDES IN NORMAL AND NEOPLASTIC TISSUES[1]

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INTRODUCTION

As part of a long-term comparative study of enzyme activity in normal and neoplastic tissues (*1*), it was observed that aqueous extracts of various kinds of mouse and rat tumors rapidly hydrolyzed peptides of α-aminoacrylic acid (dehydropeptides) (*2*). Extracts of rat and of mouse hepatomas, for example, hydrolyzed these substrates much more rapidly than did those of normal liver.

Dehydropeptides may in effect be considered as condensation products from the interaction of amino acid amides and α-keto acids (*3*). It was considered of interest, therefore, to study the susceptibility of a variety of amino acid amides to enzymatic hydrolysis in digests of normal and of cancerous tissues. The dependence of this susceptibility upon the molecular configuration of several homologous substrates and upon other experimental conditions forms the basis of this report.

EXPERIMENTAL PROCEDURE

The substrates employed were glycine amide hydrochloride (*4*), *dl*-alanine amide hydrobromide (*4*), *dl*-α-amino-n-butyric acid amide hydrobromide (*4*), *dl*-valine amide, *dl*-leucine amide, and *dl*-norleucine amide. The three last-mentioned compounds were prepared as the free base by evaporation in vacuo of ammonical solutions of the free *dl*-amino acid esters in absolute alcohol (*5*), followed by recrystallization from chloroform. All compounds were checked by total nitrogen analysis and by acid-labile amide nitrogen analysis. Aqueous solutions of the free bases were brought to pH 7.0 by additions of dilute hydrochloric acid. Stock solutions of glycine amide were 0.0125M, those of the racemic amides were 0.025M.

The digestion mixtures were composed of 1 cc. of aqueous extract of rat or mouse tissue, plus 1 cc. of buffer at the pH designated, plus 1 cc. of either water or substrate solution. The ammonia produced during the digestion was determined as described (*6*). The data were corrected for the extract blanks. The primary rat hepatomas produced by feeding rats *p*-dimethylaminoazobenzene were donated by Dr. J. White.

Where manganese chloride was used as activator, the salt was dissolved in buffer solution and the pH carefully adjusted to that desired.

In order to see whether the presence of an α-amino group in the amides was essential for enzymatic susceptibility, acetamide, chloroacetamide, and α-chloropropionylamide were included among the substrates studied.

In the figures herein, the designations of 3x, 9x, etc., refer to the volumes of water used per gram of fresh tissue in the extracts.

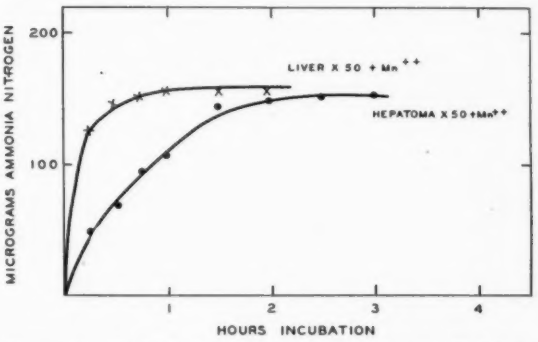

FIGURE 1.—Hydrolysis of *dl*-leucine amide at pH 8.8 in the presence of liver and of hepatoma extracts, plus glycine buffer containing manganese chloride to give a final concentration of 0.002M. Extracts equivalent to 20 mg. tissue per cubic centimeter. Ammonia N corresponding to hydrolysis of one optical form, 175 micrograms.

RESULTS

Since most of the amides studied were racemic, it was necessary to know whether one or both optical isomers were enzymatically attacked. Study of all the substrates under conditions of maximum hydrolysis showed that only one optical isomer was attacked. Representative data on a few of the racemic amides are given in table 1 and in figure 1.

Studies of the effect of pH of the digest on the

TABLE 1.—*Maximal hydrolysis of amides*[1]

Substrate	Period of incubation	Micromoles ammonia N in—	
		Kidney	Liver
	Hours		
dl-Alanine amide	2	12.0	12.0
	4	12.0	12.0
	6	12.0	12.0
dl-α-Aminobutyric acid amide	2	10.0	11.0
	4	12.0	12.0
	6	12.0	12.0
dl-Leucine amide	2	10.7	12.0
	4	11.4	12.0
	6	12.1	12.0

[1] Substrate concentrations 0.025M. Maximal ammonia N for one optical form, 12.5 micromoles. Tissue extracts equivalent to 333 mg. fresh tissue per cubic centimeter; pH, 8.8; Mn^{++}, 0.002M.

[1] Received for publication October 6, 1947.
[2] On leave from University of Brussels, Belgium.

rate of splitting of the substrates indicated that the pH at which the most rapid hydrolysis for all the amides occurred was about 8.8 to 9.0. This optimum pH was noted for liver, hepatoma, kidney, and spleen and was maintained whether added manganese was present or not. Veronal-acetate buffers were employed for the pH range 6 to 8.5, while glycine-NaOH buffers were employed in the pH range 8.5 to 10.5. Representative data are given in figures 2, 3, and 4.

The effect of substrate concentration as well as the effect of adding manganese chloride to the digests are illustrated in figures 5, 6, and 7.

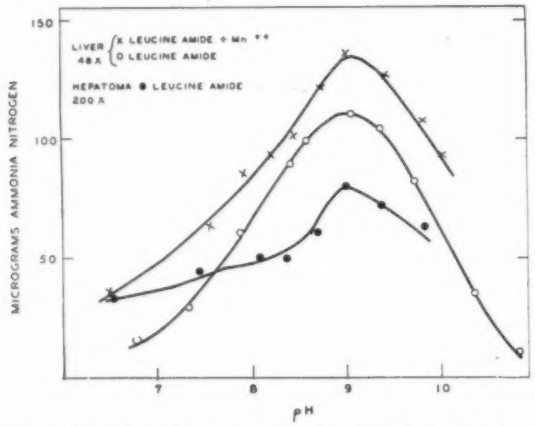

FIGURE 2.—pH-Activity curves for the hydrolysis of *dl*-leucine amide with extracts of rat liver (21 mg. tissue per cubic centimeter) and of rat hepatoma (5 mg. tissue per cubic centimeter). Incubation period for digestion with liver, 1 hour; and with hepatoma, 4 hours. Final Mn^{++} concentration in liver digests 0.002M; substrate concentration, 0.025M.

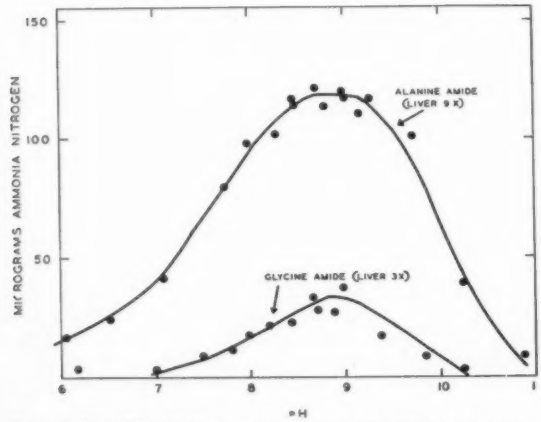

FIGURE 3.—pH-Activity curves for the hydrolysis of *dl*-alanine amide and of glycine amide in rat liver digests. Incubation period was 4 hours. Alanine amide digests contained equivalent of 111 mg., glycine amide digests 333 mg. of fresh tissue. Substrate concentration, 0.025M.

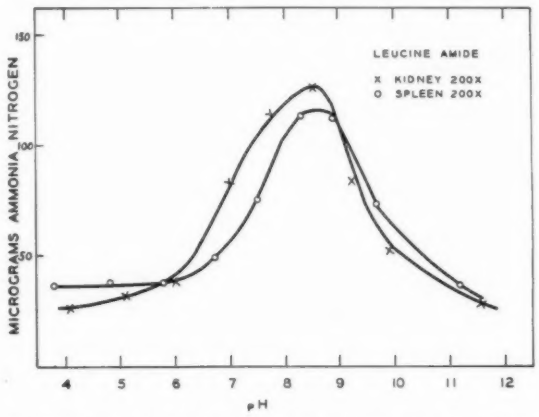

FIGURE 4.—pH-Activity curves for the hydrolysis of *dl*-leucine amide in digests with aqueous extracts of rat kidney and of rat spleen (5 mg. tissue per cubic centimeter). Incubation period 1 hour. Digests contained equivalent of 50 mg. fresh tissue. Subtrate concentration, 0.025M.

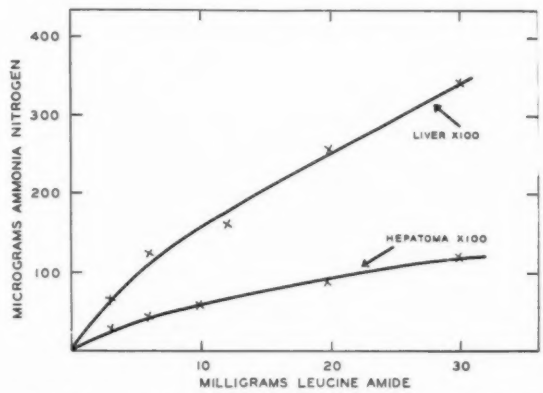

FIGURE 5.—Ammonia evolved from *dl*-leucine amide digested at different concentrations with extracts of rat liver and rat hepatoma (10 mg. tissue per cubic centimeter). pH, 8.0. No added Mn^{++} present. Incubation period, 1 hour.

A comparison of the rate of hydrolysis of the series of homologous amino acids studied under identical experimental conditions is given in table 2. A similar set of data, described in terms of percent hydrolysis of the substrates, is given in table 3.

The observation (7) that added cysteine catalyzed the splitting of *l*-leucine amide by beef-spleen catheptic preparations at pH 5.2 prompted us to repeat this experiment using *dl*-leucine amide as substrate and a dialyzed preparation of rat liver as enzyme source. No activation was observed by added cysteine or ascorbic acid in digests at pH 5.2 or at pH 7.0.

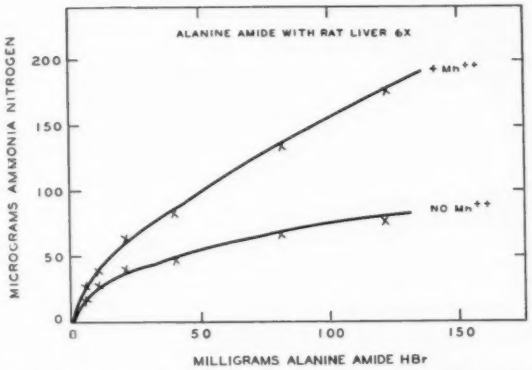

FIGURE 6.—Ammonia evolved from *dl*-alanine amide digested at different concentrations with extracts of rat liver (166 mg. per cubic centimeter) in presence and absence of added Mn^{++} at 0.002M concentration. pH, 8.0; incubation period, 2 hours.

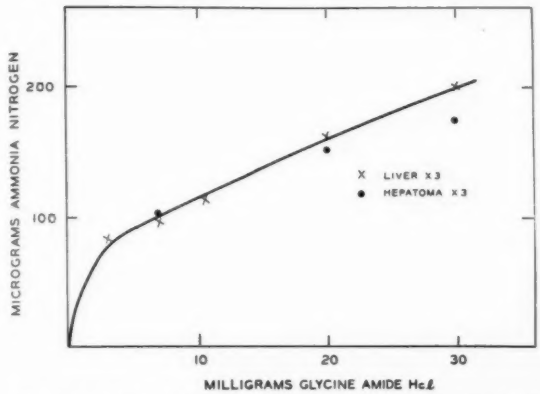

FIGURE 7.—Ammonia evolved from glycine amide digested at different concentrations with extracts of rat liver and rat hepatoma (333 mg. per cubic centimeter). No added Mn^{++}. Incubation period, 4 hours; pH, 8.0.

DISCUSSION

It is evident that all the amino acid amides studied are susceptible to enzymatic hydrolysis in aqueous extracts of all the animal tissues tested (tables 2 and 3). At least for those tissues studied, the pH optimum for this hydrolysis is at 8.8–9.0 (figs. 2–4). Behrens and Bergmann (*8*) reported that *l*-leucine amide is slowly hydrolyzed by papain, with a maximum activity at pH 4.7. It is probably due to this observation that much of the work in Bergmann's laboratory on animal tissues was performed at this low pH (*7*).

That it is the amide group of these substrates which furnishes the ammonia observed follows from several lines of evidence. If the ammonia came from the α-amino group of the substrates rather than from the amide group, it could have arisen only by the action of *d*-amino acid oxidase on the *d*-isomer of the racemic compounds. Since the ammonia is evolved equally well under anaerobic and aerobic conditions, this fact disposes of the possibility of oxidase action. Several investigators used *l*-leucine amide as a substrate in tissue preparations (*7-9*), and hence it appears most probable that it is the *l*-isomer of our racemic substrates which is hydrolyzed (table 1 and fig. 1). This would leave as products of the reaction besides ammonia, the *l*-amino acid and the *d*-amino acid amide. Neither of the last two products would be expected to yield appreciable amounts of ammonia under the conditions of our experiments.

TABLE 2.—*Comparison of hydrolysis rates of homologous amino acid amides in aqueous extracts of rat tissues*

Substrate [1,2]	Micromoles x 100 substrate hydrolyzed per milligram total N in extract of rat tissues per hour [3] in—						
	Liver	Hepatoma [4]	Kidney	Spleen	Brain	Muscle	Pancreas
Glycine amide [5]	14	27	18	18	16	3	4
dl-Alanine amide [6]	300	308	833	128	150	50	90
dl-α-Aminobutyric acid amide [6]	1,500	780	1,830	500	525	150	150
dl-Valine amide [7]	750	500		296			
dl-Norleucine amide [7]	4,000	1,600	4,000	1,630	1,050	375	500
dl-Leucine amide [7]	6,000	2,400	4,650	2,100	1,155	375	500

[1] At 0.025M, except for glycine amide which was 0.0125M, in presence of 0.002M $MnCl_2$.
[2] Acetamide, chloroacetamide, and α-chloropropionylamide are not hydrolyzed in extracts of liver and of kidney.
[3] Hydrolysis calculated on basis of susceptibility of one of two optical isomers; total available susceptible concentration=12.5 micromoles, pH 7.0. Results for *dl*-alanine amide and for *dl*-leucine amide in liver digests identical under anaerobic conditions. Boiled extracts possessed no activity.
[4] Donated by Dr. J. White. Primary tumors produced by feeding *p*-dimethylaminoazobenzene.
[5] Hydrochloride.
[6] Hydrobromide.
[7] Free base neutralized with HCl.

TABLE 3.—*Hydrolysis of amino acid amides in aqueous extracts of mouse tissues*

Substrates	Percent substrate hydrolyzed per hour in mouse tissue [1]—					
	Liver	Hepatoma 98/15 [2]	Hepatoma 12B [2]	Spontaneous mammary tumor [3]	Transplanted mammary tumor 1569A [4]	Transplanted thymoma [5]
Glycine amide	12	9	9	9	6	8
dl-Alanine amide	45	60	65	60	18	35
dl-α-Aminobutyric acid amide	100	90	90	70	30	52
dl-Valine amide	45	50	35	30	16	15
dl-Norleucine amide	100	80	90	80	45	55
dl-Leucine amide	100	100	90	90	50	60

[1] Extracts equivalent to 88 mg. tissue per cubic centimeter; pH, 7.2. For other conditions see table 2.
[2] Donated by Dr. A. B. Eschenbrenner.
[3] Donated by Dr. W. E. Heston; C3H strain mice.
[4] Donated by Dr. J. White.
[5] Donated by Dr. A. J. Dalton; A strain mice.

The effect of increasing the concentration of substrate, as shown in figures 5–7, shows how difficult it is to saturate the enzyme with substrate. The activity of the enzyme or enzymes involved in the hydrolysis of the amino acid amides is increased by addition of manganese chloride to a final concentration of 0.002M in all the digests studied. The activating effect of manganese was observed recently by Fruton (*10*).

The relative rates of hydrolysis of the homologous series of amino acid amides follow the same order in all the tissues studied (tables 2 and 3), namely, in ascending order: glycine amide, alanine amide, valine amide, aminobutyric acid amide, leucine amide, and norleucine amide. With the exception of valine amide and aminobutyric acid amide, the rate of hydrolysis of the amides increases with increasing length of the hydrocarbon side chain. The discrepancy involving valine amide and aminobutyric acid amide may possibly be due to the fact that the former possesses a branched, and the latter a straight hydrocarbon chain. On the other hand, in the case of norleucine amide and leucine amide, although the former possesses a straight and the latter a branched hydrocarbon chain, their rates of hydrolysis are not very different (table 2).

The capacity to hydrolyze the isomeric leucine amides is highest in kidney and in liver. Muscle and pancreas attack all the amides relatively weakly. Although extracts of the primary rat hepatoma hydrolyze glycine amide at a faster rate than do extracts of rat liver and alanine amide at an equal rate, the capacity of the hepatoma to hydrolyze valine amide, aminobutyric acid amide, leucine amide, and norleucine amide is appreciably less than that of liver (table 2). Glycyldehydroalanine, which might be considered as a condensation product of glycine amide and pyruvic acid, is, like glycine amide, hydrolyzed more rapidly by extracts of the hepatoma than of the liver (*2*). No data exist on dehydropeptides with alanine, valine, or leucine as the acyl amino acids. In the case of corresponding mouse tissues, the capacity of the transplanted hepatomas to hydrolyze each of the amino acid amides is not appreciably different from that of normal mouse liver (table 3).

An interesting problem which arose in the course of the present studies is whether there is a single amino acid amidase that hydrolyzes all the amino acid amides studied at different rates, or whether each substrate is hydrolyzed by a different amidase. The possibility that two or more different substrates, but not all, can be split by the same enzyme is also not excluded. The fact that the order of hydrolysis rates for the various substrates is the same in all the tissues studied (tables 2 and 3) would suggest the presence of a single amidase. On the other hand, the turn-over ratio of alanine amide to leucine amide is different in liver and kidney, for example, as well as in liver and hepatoma (table 2). A solution to the problem raised, however, cannot be readily obtained by comparing the properties of crude tissue extracts. A better approach is through the successive fractionation of a single tissue, testing the hydrolysis rate for each substrate with each successive fraction. Such a study is contemplated. That the enzyme or enzymes concerned require the presence of a free α-amino group on the substrates is shown by the failure of the corresponding α-chloro-substituted amides to be hydrolyzed (table 2).

SUMMARY

The hydrolysis of glycine amide, *dl*-alanine amide, *dl*-α-aminobutyric acid amide, *dl*-valine amide, *dl*-leucine amide, and *dl*-norleucine amide was studied in aqueous extracts of a number of normal and neoplastic tissues of the rat and the mouse. The pH of optimum activity was 8.8–9.0, and the activity was increased by added manganese chloride.

The substrates were hydrolyzed in extracts of all tissues studied, including liver, kidney, spleen, brain, muscle, pancreas, hepatoma, and a number of mouse tumors. In each tissue, the relative order of splitting of the substrates was the same, glycine amide <alanine amide <valine amide <aminobutyric acid amide <leucine amide, norleucine amide.

Glycine amide was split faster in primary-rat-hepatoma extracts than in rat-liver extracts, alanine amide was split equally fast in extracts of both tissues, while the other amides were hydrolyzed faster in extracts of liver than of hepatoma.

The corresponding α-chloro-substituted amides were not hydrolyzed.

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NOTE ON BENZOYLARGININEAMIDASE ACTIVITY IN EXTRACTS OF RAT LIVER AND HEPATOMA [1]

By JESSE P. GREENSTEIN, *chief biochemist*, and FLORENCE M. LEUTHARDT, *junior biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

Greenstein and Leuthardt reported that benzoyl-*l*-argininеamide was hydrolyzed at pH 6.9 in fresh aqueous extracts of rat liver and of the primary hepatoma induced in rat liver by the feeding of *p*-dimethylaminoazobenzene (*1*). On the basis of the original wet weight of tissue taken, there was no appreciable difference in the rate at which the normal and the cancerous tissues hydrolyzed the substrate. On the other hand, Zamecnik and Stephenson reported that at pH 5.0, aqueous extracts of the hepatoma hydrolyzed benzoyl-*l*-arginineamide at a faster rate than did extracts of the normal liver (*2*).

It occurred to us that this apparent discrepancy might be due to the fact that in the two studies the enzymatic activity was measured at a different pH. The relation of enzymatic activity to pH was therefore measured over a wide range of pH in digests of rat liver and of the primary rat hepatoma with benzoylargininеamide. The digests consisted of 1 cc. of fresh aqueous extract of normal liver or of the necrosis-free, primary rat hepatoma equivalent in each case to 333 mg. fresh tissue, plus 2 cc. of 0.14M veronal acetate buffer at various pH, plus 1 cc. of either 0.025M benzoyl-*l*-arginineamide hydrochloride or of water.[2] A third series of digests was prepared by using an aqueous extract of normal rat liver (6 mg. total N per cubic centimeter) diluted with water until the total nitrogen content per cubic centimeter was equal to that of the hepatoma (2 mg. total N per cubic centimeter). The digestion period was 4 hours at 37° C. At the end of this period, the liberated ammonia was determined as before (*1*), and the experimental values were corrected for the extract blanks. The pH of the digests was determined at each point before and after the incubation. With the exception of the digests at the highest pH ranges, namely, 7.5–8.0, where a drop of 0.1 in pH was noted, the final pH values of the digests were nearly identical with the initial values. All experiments were performed in duplicate. The data are given in figure 1.

Comparison of the hepatoma and of the liver (1) curves resolves the discrepancy between the observations of Greenstein and Leuthardt and those of Zamecnik and Stephenson. At neutral pH, there is relatively little difference in the hydrolysis of the substrate in extracts of liver and hepatoma, whereas at pH 5.0, there is a considerable difference. The optimum pH for the activity of benzoylargininеamidase in both liver and

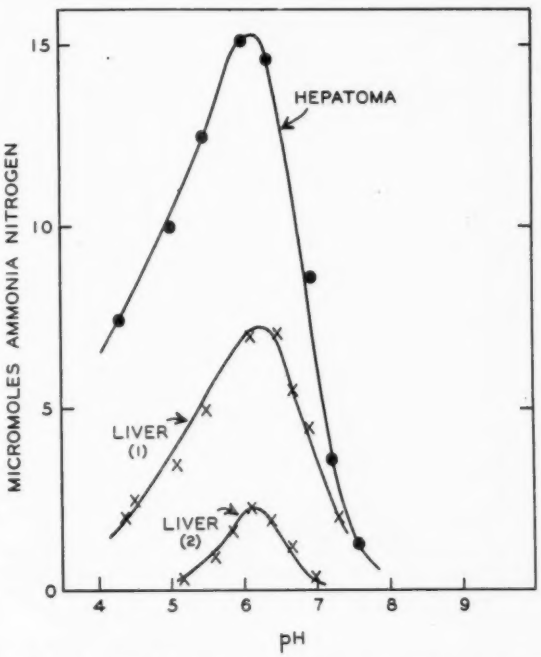

FIGURE 1.—The hydrolysis of benzoyl-*l*-argininеamide as a function of pH. Hepatoma and liver (1) curves based on equal amounts of wet weight of tissue taken; hepatoma and liver (2) curves based on equal amount of total N in the extracts.

hepatoma is 6.2, and at this pH, the activity of the enzyme in the tumor is much greater than in the normal liver. On the alkaline side of this optimum pH, the activity in the hepatoma extracts falls off rapidly with increasing pH, so that from pH 7 on, the difference in activity between liver and hepatoma is almost inappreciable. At the optimum pH and at more acid ranges, there is no question but that the hepatoma is more active than normal liver in hydrolyzing benzoylargininеamide. This difference is further emphasized by a comparison of the hepatoma and liver (2) curves based on equal total nitrogen contents in the respective extracts.[3]

Fruton and Bergmann reported that the activity of their beef-spleen catheptic preparations in hydrolyzing benzoylargininеamide was optimal at pH 4.7 (*3*). It is difficult to compare their findings with ours, since Fruton and Bergmann were work-

[1] Received for publication October 6, 1947.

[2] The primary hepatomas were originally induced in rats by the feeding of *p*-dimethylaminoazobenzene and were donated by Dr. J. White.

[3] The activity of extracts of the transplanted rat hepatoma 31 on any basis was found to be greater than that of normal liver (*1*).

ing with an acidified and isolated protein fraction, whereas we have utilized the complete tissue extract.

In the earlier report, Greenstein and Leuthardt (*1*) described an inhibitory action of added inorganic phosphate at pH 6.8–7.0 on the hydrolysis of benzoylarginineamide in tissue extracts. In view of the steep character of the pH-activity curve (fig. 1), whereby it is conceivable that relatively small changes in pH during the digestion would be reflected in large changes in enzymatic activity, it was considered desirable to check certain of the earlier observations on the effect of phosphate, while making every effort to maintain a nearly constant pH in the digests. Data on rat-liver extracts are given in table 1.

The data in table 1 confirm our earlier observations. The very slight difference in the final pH of the digests with and without added phosphate would amount to about 0.2 micromoles of ammonia nitrogen (fig. 1). With this correction, the value in table 1 for the micromoles of ammonia nitrogen hydrolyzed from the substrate in the presence of phosphate becomes 1.5 instead of 1.3. The striking inhibitory action of added phosphate in this case takes on added interest in view of the equally striking activating influence of added phosphate on the desamidation of glutamine (*4*). Care in the use of phosphate as a component in buffer systems for enzyme studies is indicated.

TABLE 1.—*Effect of added inorganic phosphate on the activity of benzoylargininеamidase in rat liver extracts*[1]

Digest	Initial pH	Final pH	Micromoles ammonia N
No added phosphate	6.65	6.61	7.7
With added phosphate	6.66	6.64	1.3

[1] Digests and conditions as in text. Rat-liver extract equivalent to 333 mg. fresh tissue per cubic centimeter. Sodium phosphate dissolved in the veronal buffer to give a final concentration in the digest of 0.075 M. Experiments were performed in duplicate.

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EFFECTS OF METHYLCHOLANTHRENE IN PREGNANT MICE[1,2]

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INTRODUCTION

Very few studies have been reported on the effects of carcinogenic compounds in pregnant animals or on embryos. Law (*1*) injected an olive-oil solution of 1, 2, 5, 6-dibenzanthracene into the amniotic fluid of 14-day mouse embryos (i. e., gestation two-thirds completed). The embryos were hybrids between two partially inbred strains (Caracul and P). He found that abortions and premature birth were the common result; and of the surviving young, the great majority developed lung tumors by the age of 200 days. Regular development of lung tumors also followed intraperitoneal injections into day-old mice.

Wolfe and Bryan (*2*) injected large daily doses of carcinogens (chiefly 1, 2, 5, 6-dibenzanthracene), dissolved in sesame oil, subcutaneously into rats throughout pregnancy. In most cases, placental hemorrhage began about midpregnancy, followed by death and resorption of most but not necessarily all embryos.

Greene (*3*) reported early tumor development from embryonic tissues impregnated with methylcholanthrene and grafted subcutaneously or elsewhere into adult hosts of the same strain or even other strains.

In this laboratory, thousands of mice of various strains have been given a single subcutaneous injection of 1.0 mg. of methylcholanthrene dissolved in 0.1 cc. of sesame oil at the age of 60 days. At this age a majority of the young females are sexually mature and may be already pregnant. Strong (*4, 5, 6*) has reported on mutations and other changes in the descendants of such mice, treated generation after generation. Reproduction has not been seriously interfered with by this treatment, although some evidence of excessive embryonic mortality has been noted. Moreover, carcinogenesis in embryos has not been observed following subcutaneous injection of the mothers. It seemed desirable to investigate further the effects of methylcholanthrene during pregnancy, both as to possible genetic changes and neoplasia.

Reciprocal crosses between mice of several inbred strains were consequently started, and the females were injected when pregnant. The hybrid offspring might be expected to disclose maternal transmission such as the milk influence, as well as genetic changes in the form of somatic mosaic effects.

MATERIALS AND METHODS

Since this was essentially an exploratory study, simple methods were chosen. In view of the indirectness or remote location of subcutaneous injections into the mother and the drastic results of intra-amniotic injection, we decided to inject once intraperitoneally at about the time of conception. By this means, it was hoped that the embryos would be exposed throughout pregnancy to some methylcholanthrene or its decomposition products in the blood plasma. The dose was 1.0 mg. in 0.1 cc. of sesame oil.

The strains of mice used were A, C3H, JK, L, C57, and I. The origins of these strains are discussed by Strong (*7*). Genetic formulas for known genes in these strains are given in table 1.

Table 1.—*Genetic formulas for mice of various strains*

Strain	Genetic formulas[1]						
A	$\frac{a}{a}$	$\frac{b}{b}$	$\frac{c}{c}$	$\frac{P^+}{P^+}$	$\frac{D^+}{D^+}$	$\frac{S^+}{S^+}$	$\frac{Se^+}{Se^+}$
C3H	$\frac{A^+}{A^+}$	$\frac{B^+}{B^+}$	$\frac{C^+}{C^+}$	$\frac{P^+}{P^+}$	$\frac{D^+}{D^+}$	$\frac{S^+}{S^+}$	$\frac{Se^+}{Se^+}$
JK	$\frac{a}{a}$	$\frac{b}{b}$	$\frac{C^+}{C^+}$	$\frac{p}{p}$	$\frac{D^+}{D^+}$	$\frac{S^+}{S^+}$	$\frac{se}{se}$
C57	$\frac{a}{a}$	$\frac{B^+}{B^+}$	$\frac{C^+}{C^+}$	$\frac{P^+}{P^+}$	$\frac{D^+}{D^+}$	$\frac{S^+}{S^+}$	$\frac{Se^+}{Se^+}$
L	$\frac{A^w}{A^w}$	$\frac{B^+}{B^+}$	$\frac{c^{ch}}{c^{ch}}$	$\frac{P^+}{P^+}$	$\frac{D^+}{D^+}$	$\frac{S^+}{S^+}$	$\frac{Se^+}{Se^+}$
I	$\frac{a}{a}$	$\frac{b}{b}$	$\frac{C^+}{C^+}$	$\frac{p}{p}$	$\frac{d}{d}$	$\frac{s}{s}$	$\frac{Se^+}{Se^+}$

[1] A^w, white-bellied agouti; c, albino; p, pink-eyed-dilute; +, wild-type allele; s, piebald; a, non-agouti; b, brown; c^{ch}, chinchilla; d, dilute; se, short-eared

The crosses most employed were A×C3H and C3H×JK. The hybrids are heterozygous for three color genes. Because chromosomal aberration might be expected to occur in the embryos of the treated mothers, these genes afford some opportunity for its recognition by mosaic effects.

The parent mice used were relatively young (2–5 months) and healthy. They were given the customary care of this laboratory as described in previous papers. Daily observations (except Sunday) were made for vaginal plugs and indications of pregnancy (including the "placental sign"), as well as for parturition. Most of the F_1 offspring born within 90 days after the single injection of methylcholanthrene into the females were raised. After 4 months, the treated females were separated from the males and observed weekly for tumors.

Controls (uninjected) were handled in the same way as the females of the treated series. In some instances, a female to be injected produced a litter before treatment, and this record was added to the control series. All young born of untreated females were killed soon after birth.

No attempt was made to check the effect of injecting sesame oil without methylcholanthrene, but, as indicated in the general discussion, this omission is probably not serious.

[1] Received for publication September 23, 1947.
[2] This experiment was made possible by grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

As a separate brief test, several females of various strains were injected intraperitoneally in late pregnancy (twelfth to nineteenth day). In all cases, there were normal parturition and lactation, and the young grew well.

In another brief test, several of the early injected females were killed at intervals of different length after injection, and the peritoneal cavity was examined under ultraviolet light to estimate the persistence of the methylcholanthrene. In most cases, fluorescence was greatly diminished or absent by the third week.

RESULTS

The present report includes the results obtained with the treated mice and the F_1 hybrids up to the end of 1 year after the original injection. Since many of the animals are still alive, complete statistics on tumor incidence in F_1 cannot be given, but the principal conclusions to be drawn seem apparent already.

For about a week after the injection of methylcholanthrene, no visible effect was observable. About the middle of pregnancy, however, there was frequently a discharge of dark blood from the vagina. The amount could rarely be considered serious hemorrhage, but it was definitely in excess of the usual traces at this time, the "placental sign." Where the blood flow was observed, pregnancy generally ended in resorption of the embryos, although a few young were born. Apparently there were no premature births.

Table 2 shows the contrast between strains in the effect of treatment. Females of strains C3H and A have a high frequency of embryonic death, especially in the first pregnancy, while strain JK females show practically no difference from the control. Reduced numbers of young per litter continued for subsequent pregnancies in females of strains C3H and A, though complete loss of embryos was uncommon.

In spite of the great mortality of embryos, only half a dozen young were born dead or deformed. The difference between the treated and the control females in this respect was apparently insignificant. In general, the F_1 mice have been vigorous and prolific. The sex ratio has not been significantly changed from 1:1. Mosaic color effects failed to appear. One male C3H×JK from a litter of two showed a peculiar hair whorl at the

BREEDING RESULTS

STRAIN GROUP		Order of Cycles	No. of Cycles	Missed	Abort.	1	2	3	4	5	6	7	8	9	10	11	12
C3H	CONTROL	1	21														
		2	17														
		3	15														
		4	6														
		ALL	59					2	9	14	15	11	4	3	1		
	MCA INJECTED	1	18														
		2	16														
		3	12														
		4	4														
		ALL	50	2	23	1	4	2	6	6	3	2	1				
JK	CONTROL	1	15														
		2	9														
		3	7														
		4	4														
		ALL	35	3	3	2	1	2	4	4	2	4	3	3	3	1	
	MCA INJECTED	1	14														
		2	13														
		3	6														
		4	3														
		ALL	36	7	1	1	2	2	1	4	2	3	4	5		4	
A	CONTROL	1	40														
		2	18														
		3	10														
		4	7														
		ALL	75	10	1	1	1	3	10	10	14	11	11		3		
	MCA INJECTED	1	30														
		2	22														
		3	12														
		4	3														
		ALL	67	22	12	2	2	11	6	1	8	2	1				

MCA, methylcholanthrene; Cycle, reproductive cycle as indicated by vaginal plug and/or pregnancy. Missed, vaginal plug observed, but no sign of pregnancy. Abort., signs of pregnancy observed but no litter produced. 1, 2, etc., number of young in each litter. The statistics for each group are summarized by heavy line giving the range of distribution and divided to show quartiles; the median value is indicated by a pyramid.

end of the nose. Extensive progeny tests demonstrated that the trait was not inherited.

Carcinogenic Action on Injected Mice

Mammary adenocarcinomas occur spontaneously in many females of strains C3H and A by the age of about 1 year. The frequency in our intraperitoneally injected mice was clearly not increased (table 3). While this type of tumor is not found in untreated JK females, 2 cases did appear among the 14 injected female mice of this strain. A similar observation on JK female mice was made by Strong and Smith (*8*) following the subcutaneous injection of methylcholanthrene.

Table 3.—*Mortality of female mice injected intraperitoneally at the age of 2-5 months with 1 mg. of methylcholanthrene in 0.1 cc. of sesame oil*

Strain	Number of mice injected	Died, no tumor		Mammary tumors		Other tumors, etc.		Living at 1 year
		1-6 months	7-12 months	1-6 months	7-12 months	1-6 months	7-12 months	
A	27	1	5	2	3	[1] 1, [2] 1	[3] 5	9
C3H	16	3	1	0	5	[2] 2	0	5
JK	14	3	0	0	[4] 1, [4] 1	[3] 1	[2] 2	6
C57	8	1	0	0	0	[1] 1	0	6
L	2	0	0	0	0	0	2	0
I	5	1	3	0	0	0	0	1

[1] Subcutaneous fibrosarcoma at site of injection.
[2] Leukemia.
[3] Lung pearls present.
[4] Also a bronchiogenic carcinoma of the lung.
[5] Myxoma.

Subcutaneous injection of this dose of methylcholanthrene readily induces local fibrosarcomas in mice of strains C3H, C57, L, and A, but much less so in JK and I. With the same dosage, but intraperitoneally, we do not find the same relative susceptibility, and most of the tumors that have appeared seem rather benign and late.

The animals surviving a year following the injection of methylcholanthrene died or were killed shortly thereafter. Most noteworthy is the great frequency of small lung tumors ("pearls") in females of strain A. In a few cases, the lungs were almost entirely filled with these tumors. A few tumors of this kind have also been found in many surviving females of all the other strains. Next most common have been hemangiomas, mostly small, in the wall of the uterus or intestine. Several females of strains A and C57 showed also one ovary enlarged and red (cystic).

The only tumors observed thus far in the F_1 mice have been mammary adenocarcinomas in females having C3H or A mothers (milk influence?) and in two females from JK mother × C3H father. Of 75 F_1 males killed at the age of 1 year, not 1 had a tumor of any kind. The methylcholanthrene treatment of the mothers seems to have had no definite carcinogenic action on their young.

Discussion

The most definite positive effect of the injection of methylcholanthrene into pregnant mice has been embryonic death. The surviving hybrid offspring seem to have been relatively unaffected.

What can be the physiologic basis for the embryonic death? It seems quite comparable with the results obtained in rats by Wolfe and Bryan (*2*), using other carcinogens dissolved in sesame oil. They found no disturbance following injection of plain sesame oil and concluded that the hydrocarbons had a specifically toxic action on gestational tissues. They were unable to relate this effect closely to carcinogenicity since even 1,2-benzanthracene (a weak carcinogen) produced it.

The toxic action, if any, is evidenced by uterine hemorrhage and death of the embryo, but a direct action on the embryonic tissues seems unlikely. The mother appears to be the primary target, from the results of the reciprocal crosses of C3H × JK. The female hybrid embryos are of identical genetic constitution, as are the males, except for possible sex-linked genes. Nevertheless, those in C3H mothers have high mortality while those in JK mothers do not. It may be significant that mice of the strains in which this high embryonic mortality is found are also quite susceptible to local tumor induction with subcutaneously injected methylcholanthrene.

The uterine disturbance produced by these carcinogens seems suggestively similar to the effects of vitamin A deficiency, as reported for the rat by Mason (*9*). Hemorrhage such as we have noted also occurs with vitamin A deficiency, and some of the embryos may survive. Several investigators (*10–13*) have shown that carcinogens such as methylcholanthrene and dibenzanthracene can produce depletion of vitamin A stores in the liver of rats and mice. This effect seems to be fairly specific and not produced with phenanthrene or other noncarcinogenic hydrocarbons. It seems possible, therefore, that disturbance of vitamin A metabolism of the mother during critical periods of gestation is the basis for the embryonic mortality in our experiment, and that in the JK mothers such a disturbance is less easily induced than in C3H or A mothers.

However, definite signs of vitamin A deficiency have not been observed either in the mother mice or the surviving offspring. Perhaps an effect on the corpus luteum is involved, since this gland also stores carotenoids. The problem therefore needs further investigation. Whether the methyl-

cholanthrene (or its metabolites) ever reaches the embryos is not clear from the present experiment, although mutations in subsequent generations, which will be reported elsewhere, indicate this probability.

In regard to the carcinogenic action of the methylcholanthrene by intraperitoneal injection, we wish to emphasize the difference from results with subcutaneous injections. By the latter route, local fibrosarcomas are readily induced with the dosage that we have employed, while intraperitoneally it is much less potent. Carruthers (*12*) obtained visceral tumors in Swiss mice with repeated intraperitoneal injections, but no comparison with the subcutaneous route was made. In this connection, the relatively rapid disappearance of fluorescence from the peritoneal cavity is noteworthy. Subcutaneously injected methylcholanthrene is recognizable by fluorescence for a far longer period, four or five times as long.

SUMMARY

Female mice of several strains were mated with males of other strains and injected intraperitoneally at about conception with 1 mg. of methylcholanthrene in 0.1 cc. of sesame oil.

High embryonic mortality resulted in females of strains C3H and A, but not in JK. Since the embryos in reciprocal crosses are genetically the same, the immediate action of the carcinogen must be on the mother rather than directly on the embryo. In view of other studies showing disturbed metabolism of vitamin A after carcinogen injections, it is suggested that this may be the main basis for the embryonic mortality. Evidently the females of strain JK are more resistant to this disturbing action of methylcholanthrene than are females of strains C3H or A.

Intraperitoneal injection of methylcholanthrene was found much less effective in producing tumors than was a subcutaneous injection of the same dose.

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PROCEDURE FOR THE FIXATION, STAINING, AND MOUNTING OF WHOLE MOUNTS FROM TISSUE CULTURES GROWN IN CARREL D 3.5 FLASKS[1]

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INTRODUCTION

In studies of tissue cultures in this laboratory over a period of years, nearly all cultures have been grown in Carrel D 3.5 flasks. While living cells can be routinely photographed in such flasks, by using a 16-mm. or 8-mm. objective and working at a magnification of from 200 to 1,000 on the camera previously described (*1*), it has often been necessary to obtain fixed and stained preparations of such cultures. Procedures have already been described by which fixed and stained preparations have been obtained from hanging- or lying-drop slide cultures explanted from such Carrel D 3.5 flask cultures (*2*). Such methods have proved to be of value in the study of the Golgi apparatus and are necessary in a detailed study of such minute structures as chromosomes or mitochondria. However, a procedure has been needed by which a culture growing under control conditions in a Carrel flask could be fixed, stained, and mounted as a whole-mount preparation for general microscopic examination without the disturbance of the culture, change in cell relations, and loss of control conditions necessarily associated with transfer of the cells to slide culture.

The method finally worked out and in routine use in this laboratory has proved so useful that a description of the procedure and equipment are given herein.

EXPERIMENTAL PROCEDURE

KILLING AND FIXATION

At least 12 hours before fixation and preferably immediately after the last fluid change, cultures to be fixed are selected and placed in flask racks (*3*) specially reserved for use with fixing fluids. The cultures are then incubated as usual. At the time of fixation, one rack is removed from the incubator. Without removing the flasks from the rack, the rubber stoppers are rapidly removed from the flasks, and the whole rack is inverted so that the fluid culture medium from all flasks is run out into a sink or waste jar. Freshly prepared formalin-dichromate,[2] warmed to 38° C., is at once added to fill all flasks, and the rack is returned to the incubator. This addition of fixing fluid to the culture is completed as rapidly as possible to avoid cell changes which might result from handling or cooling. This procedure should take less than 3 minutes after removal of a rack from the incubator. The fixing solution is allowed to remain on the cultures for about 10–15 minutes in order to penetrate the thickness of the plasma clot and kill all cells. The cultures are then removed from the incubator. All subsequent operations are carried out at room temperature unless otherwise noted. Immediately after removal from the incubator, the fixing solution is changed and again changed after periods of 10, 15, 15, 30, and 60 minutes, and 6, 24, and 48 hours, respectively.

REMOVAL OF CULTURE FROM FLASK

Culture flasks are then rinsed out with tap water, filled to the neck with water, and the circular sheet of fibrin clot with its contained culture is dissected off from the floor of the flask.

This loosening of the plasma clot is usually done by means of the special spatula previously described (*3*). With the relatively sharp edge of the spatula kept against the floor of the flask, the clot is pushed loose from the glass with rapid, short, sharp strokes of the spatula, starting at the flask throat and working evenly across the width of the flask. The floor of the flask is meanwhile held inverted and at an angle of about 30° with the horizontal, so that once the edge of the clot has been rolled loose from the floor near the throat of the flask, its own weight tends to pull it away from the glass and roll it over the free surface of the plasma sheet still adherent to the flask.

When the culture has been hardened in the flask, the plasma is so brittle that it cannot easily be worked loose in this way. Instead a special clock-spring spatula[3] (fig. 1) is used. This is done by holding the flask with its floor down and horizontal, while the cutting edge of the spatula is pushed between the plasma and the glass. Long, slow, sweeping strokes are used, and care is taken to withdraw the spatula before changing its position laterally. After either method of dissection, the plasma clot and its contained culture, together with the overlying fluid, are poured and shaken out of the flask. This clot and culture with the supernatant wash water are then poured into labeled, 4-ounce, wide-mouth specimen bottles with a throat diameter of at least 40 mm. to allow easy

[1] Received for publication August 12, 1947.

[2] This solution consists of 10 to 12 cc. of formalin (37 percent formaldehyde gas) made up to 100 cc. with 3 percent potassium dichromate. Both formalin and potassium dichromate are chemically pure, and solutions are made up in distilled water. The solution must be prepared just before use and is discarded when it shows any signs of darkening.

[3] This instrument is ground (on an emery wheel) to shape from steel clock spring of 10-mm. width and ½-mm. thickness, while edges indicated in figure 1 are first rough sharpened on the emery wheel then polished fairly sharp on a fine Arkansas oil hone.

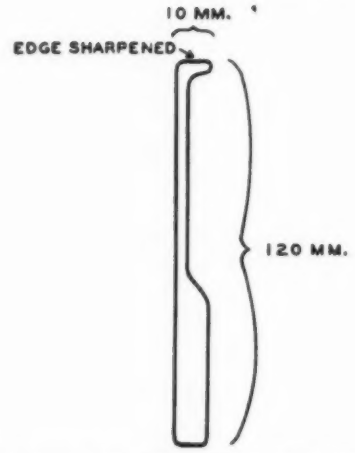

FIGURE 1. Spatula used for dissecting out hardened culture from Carrel D 3.5 flasks.

insertion of the cultures. The water is drained off, and 3 percent potassium dichromate is added.

HARDENING

Cultures are hardened 5 to 8 days in 3 percent potassium dichromate solution at 4° C. The solution must be changed at any sign of discoloration. Because of formalin carried over from the formalin-dichromate fluid, this may necessitate two changes the first day, one the second, and possibly one the third day.

WASHING

After hardening, the specimen bottles are uncorked and covered with a layer of medium-mesh surgical gauze held over the mouth of the bottle by a rubber band. The bottles are then filled with water, inverted under water, and placed in a sloping position on the washer.[4] They are washed about 24 hours in tap water, after which each culture is transferred to a labeled low Stender dish.

Tissues to be stored before staining are passed up through 35, 50, and 65 percent alcohols, about 10–15 minutes in each, and finally stored in 80 percent alcohol. When needed for staining, they are passed down through 65, 50, and 35 percent alcohols and then placed in a mixture consisting of 2 parts of water, 1 part of alcohol, and 1 part of glycerin. Tissues to be stained without storage may be transferred to this glycerin-alcohol mixture directly after washing. They must be held under the surface in this solution as they have a tendency to rise and float on the surface, which would allow the top layer of cells in the culture to dry out and shrink.

[4] The general design of this washer is shown in figure 2.

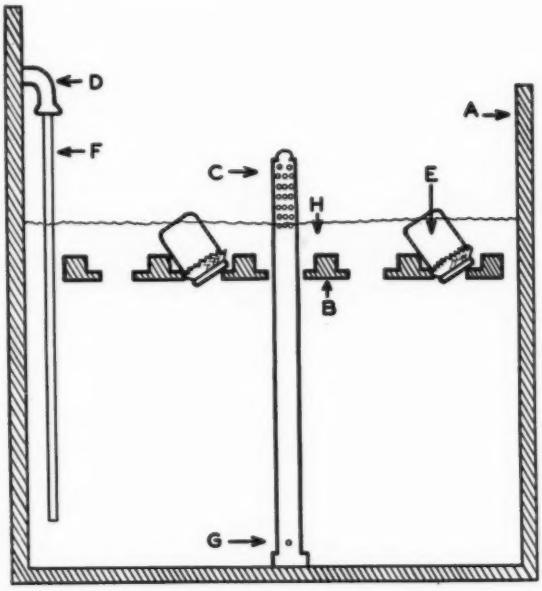

FIGURE 2.—Cross section of tissue washer. Whole washing rack is contained within a standard stone laboratory sink about 36 x 18 x 18 inches in size. The wooden rack supporting the specimen bottles is anchored into the sink so that it does not float. The water inlet is well away from the specimen bottles so that air coming in with the water does not rise under the bottles, filling them with air and causing them to float free. The 2 small (¼-inch) drain holes near the bottom of the drain pipe carry off the heavier waste fluid from the bottles. The bottles are sloped so that air bubbles from the water do not readily enter them.

STAINING

For staining, cultures are handled in lots of about 20. Cultures are stained 2 hours in a special iron hematoxylin.[5] A layer of hematoxylin not less than 1 cm. deep in each dish is satisfactory. At the end of 2 hours the hematoxylin is drained off, filtered, and returned to the stock bot-

[5] This solution is made up as follows:
Solution A, 0.9 gm. hematoxylin, and 25 cc. absolute alcohol; solution B, 15.0 gm. ferric ammonium sulfate, crystalline reagent, and 50.0 cc. distilled water; solution C, 15.0 gm. ferrous sulfate, crystalline reagent, and 50.0 cc. distilled water; solution D, 25 cc. absolute alcohol, and 50 cc. glycerin.
These solutions are freshly prepared at room temperature. When ready, A and B are mixed with shaking, and the mixture is allowed to stand in an open flask for 2 hours at room temperature. Solution C is then added and mixed. Thirty minutes later, solution D is added, and the entire solution mixed and kept overnight in a glass-stoppered bottle. When kept at room temperature, this solution is good for 30 to 60 days for staining of whole-mount tissue cultures from Carrel D flasks but should be discarded when it begins to give the fibrin matrix of the culture a diffuse, often brownish stain. For sections, the stain may be used much longer as described by Earle (4), and by Lillie and Earle (5).

tle. The Stender dish containing the culture is rinsed with distilled water, drained, and filled with distilled water. If the work is done rapidly, by the time all cultures have been rinsed and water has been added, the water in the first culture is deeply stained with hematoxylin soaked out from the culture and is ready to be drained. After draining, fresh water should be added. Washing each culture with distilled water is continued as rapidly as possibly through several changes of water. With each washing, the discoloration of the wash water is slower and more faint. The cultures are carried through 2 washings of 15 minutes each after the last wash water appears entirely colorless. The cultures should not be left in water overnight, as the hematoxylin stain deteriorates. If necessary, it is much better to pass the cultures up through the alcohols and leave them in one of the special dehydrating mixtures.

Dissection of the Culture

During the later stages of washing, since fluid changes are less frequent and consequently require less of the operator's attention, the cultures may be trimmed. Each culture in turn is poured, under water, into a shallow petri dish; and then, under a very low power objective of the dissecting microscope, excess clot is cut away from the edge of the culture. A free edge of several millimeters of cell-free clot should be left at the periphery of the culture since, if this is not done, light enters the objective from around the cut edge of the clot, when the peripheral cells of the culture are being examined microscopically. This extraneous light floods the objective and so obscures the clarity of the image.

Where cell growth has been extensive along both fluid and glass interfaces of the fibrin clot, it is usually advisable to split the culture lengthwise so that one-half the culture may finally be mounted with the cells of the fluid interface up. When the central explant area of the culture is so thick that the cover slip will rest upon it, this area may be dissected out and discarded.

If a finer dissection is desired for any special purpose, it may be carried out when the culture is in toluene and is consequently more transparent.

Dehydration, Clearing, and Mounting

The cultures are dehydrated through 35, 50, 65, 80, and 95 percent, and absolute alcohols and then passed through six clearing mixtures[6] at least 10 minutes being allowed for each. When ready to mount, a rectangular paper cell[7] is placed on a clean 25- x 75-mm. slide, and an excess of clarite solution[8] is placed in the chamber so formed. Each half of the culture is then removed from the toluene, placed in the clarite, and covered with the clarite solution as rapidly as possible to prevent drying. The clumps are then arranged and oriented with their correct surfaces up, in the clarite solution in the paper cell. A 24- x 50-mm. No. 1, or preferably No. 0, cover slip moistened with a little clarite is then slowly lowered and slid onto the paper-cell preparation in such a way as to drive out all air from the preparation. When the paper cell is so closed, care should be taken to see that a large drop of clarite is left covering the open aperture in the paper ring.

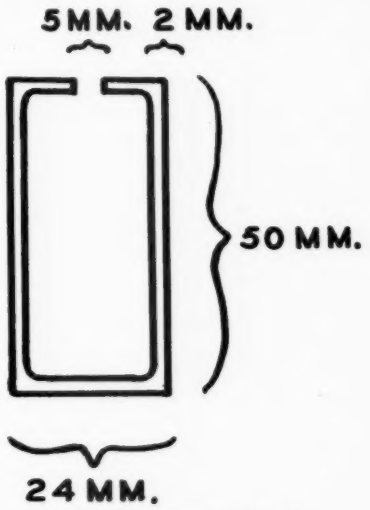

Figure 3.—Paper cell used.

Drying and Hardening of Slides

The slides are then placed flat on a standard slide-drying hot plate held at about 40° C. As the clarite hardens, it loses solvent and shrinks and so sucks in additional clarite from the clarite drop at the aperture of the chamber. Each slide is examined daily, and additional clarite is added when necessary at the chamber aperture. Air bubbles are removed with a needle dipped in toluene. By examining with this frequency, relatively little

[6] Clearing solutions used are as follows:

Solution designation	1	2	3	4	5	6
Percent absolute alcohol	70	50	25	0	0	0
Percent acetone	25	25	25	25	10	0
Percent toluene	5	25	50	75	90	100

[7] For use with a 25-x-75-mm. slide and 24-x-50-mm. cover slip, a cell of the shape and dimensions shown in figure 3 has given best results. The open end of the cell facilitates elimination of air and introduction of additional clarite during drying. Such cells have been cut from cardboard library-record cards which have a thickness of 0.22 mm. It would be advantageous to use slightly thinner stock, however, in case thinner cultures, such as occur in "roller tube" tissue-culture preparations, are being mounted.

[8] This solution consists of 200 gm. of clarite (as sold by the Neville Co., Pittsburgh) dissolved in 100 ml. of toluene.

trouble is experienced from sucking air into the preparation.

At the end of about 4 days, the slides have completed a necessary preliminary hardening. They are then examined, returned to the hot plate, or to an incubator at about 38°, and two special lead weights [9] are gently lowered onto the cover slip. After adding the lead weights, the slides are examined and minor adjustments made periodically, once a day for several days, then less and less frequently, until finally they are examined only about once a week. The slides are considered dry and are cleaned and permanent labels attached in about 2 months. All such thick preparations must of course be stored flat.

GENERAL CONSIDERATIONS

Choice of Fixatives

In working out this procedure for the preparation of stained slides from tissue cultures grown in Carrel flasks, an attempt was made to obtain routine preparations with as little change as possible in general cell design, in cell relationships, and in nuclear design. For this reason, care was taken to fix the cultures with an agent warmed to 38°, with as little agitation and chilling as possible, and without previous washing with a saline solution or with water.

An extensive number of fixing solutions were studied with respect to their action on tissue cultures. Solutions containing active protein precipitants such as Bouin's fluid and those containing mercuric salts such as Zenker-formol were quickly eliminated since they caused such a severe precipitation (probably protein) within the fibrin clot that cell structure was obscured. While this effect could largely be prevented by a preliminary washing of the culture in isotonic saline, the attendant hazard of inducing cell change led to abandonment of these types of fixatives.[10] Even when precipitation within the fibrin clot by the fixing solutions was prevented by preliminary rinsing of the culture, a fine, lightly staining granulation was often produced within the nucleus. This granulation seemed to arise from the precipitant or coagulant action of the fixative and complicated the interpretation of nuclear structure. Osmic fixatives were abandoned because trouble was experienced in getting consistent staining after their use. The formalin-dichromate fluid here recommended caused none of these difficulties.[11]

The early results with this material were erratic, and the cells often showed severe lateral shrinkage, particularly when the cultures were sectioned. When used for whole mounts, its effects were much more uniform. Another source of erratic behavior was eliminated by making up the fixing solution from chemically pure formalin and dichromate. (Bottles of formaldehyde showing severe precipitation are not used since the concentration of active formaldehyde gas would be reduced.) Properly prepared, the fixing solution showed a pH of about 3.7 as determined by the glass electrode.

The mixture should be discarded as soon as it begins to show appreciable discoloration. Deterioration is greatly accelerated at 38° so that the fixative is used at this temperature for only a short period to kill the cells, the fixation and hardening being completed at 4° to retard further deterioration of the solution.

Effect of pH on Fixation

In view of the recognized effect of pH on the action of a fixative and in view of the rather frequent use of "neutral" formalin for fixation, studies were made of formalin-dichromate mixtures acidified by addition of a little chromic or acetic acid, and partially neutralized by the addition of potassium hydroxide. The shift of pH to a more acid level did not improve fixation and caused some artifactitious granulation within the cell nuclei, while the shift of pH to the range 5–8, resulted in a progressively more severely washed out nucleus. Use of such neutralized solutions, therefore, seems contra-indicated where any accuracy of nuclear fixation is desired.

The capacity of the usual Carrel D 3.5 flask is about 10.5 cc., of which about 1 cc. is taken up by the solid-culture medium, which is heavily buffered to about pH 7.3 to 8.4. When the culture is drained of fluid and fixing solution is added to fill the flask, the normal pH 3.7 of the fixing solution is carried to far more alkaline levels by the buffering action of the solid-culture medium. Actual experiment showed that the final pH is sufficiently alkaline to produce a washed-out appearance of the nuclei. To eliminate this, each flask culture was

[9] The lead weights are easily made by casting in a wooden mold made by clamping together two 2-x-4-inch boards, so that the whole makes up a 4-x-4-inch wooden strip of any convenient length. With an ordinary wood bit, holes ¾ inch in diameter and 3 inches long are then drilled along the seam between the two boards. These holes are filled with melted lead, and after cooling the lead cylinders are taken out by unclamping the boards. Each weight is then clamped in the chuck of a lathe, and one end is faced off smooth so that when placed upright on the cover slip the lead cylinder rests squarely on the cover slip. Each lead weight weighs about 260 gm.

[10] Where for any special reason it is desired to use Zenker-formol, it is probably better first to give the cultures a short wash in warm formol-dichromate rather than saline and then replace this with Zenker-formol.

[11] Culture flasks to which potassium dichromate has been added must be carefully recleaned before being returned to use. This cleaning is done by heating them in a mixture of 25 percent nitric and 75 percent sulfuric acids on a warm steam bath in a hood for about 2 days, then rinsing with running water, and finally transferring to the nitric-sulfuric acid kettles previously described (6). This preliminary acid cleaning before the culture flasks are placed in the acid kettles serves to eliminate practically all chromium from the flasks and so to keep from building up a possibly toxic concentration of heavy metal salts in the acid kettles. When the culture flasks have contained carcinogen, the flasks must be handled most carefully before cleaning in order to avoid the possibility of disseminating the carcinogen about the laboratory.

subjected to repeated changes of the formol-dichromate solution so as to wash out the alkaline buffer of the culture medium and rapidly bring the pH to a level satisfactory for fixation.[12]

Hardening

The use of 3 percent dichromate to harden the cultures further after fixation is probably not essential, but it gives the cultures substantially better consistency and possibly lessens distortion during dehydration and mounting. Cultures not yet completely hardened are less brittle and are consequently easier to remove from the flask following fixation and before chroming. The working procedure outlined, therefore, follows this order.

Washing

If, through oversight, the cultures have been allowed to remain in the fixing solution so long that the brownish-yellow color of the fixing solution cannot be washed out, this color may be removed before staining by treating 5 minutes with ¼ percent potassium permanganate, washing the culture a short while, treating 5 minutes with 5 percent oxalic acid, and then washing thoroughly before staining. Of a number of methods used, the most satisfactory results were obtained by using the washer and washing procedure as described. Since the specific gravity of the fixing solution is greater than that of water, the fixative rapidly settles out through the gauze on the specimen bottle and into the tank of running water, from which it is rapidly eliminated by the drain. After about 1 hour of washing, fixed specimens are only a very pale yellow, while after 12 hours of washing they should be a clear, opalescent white. Such a correctly fixed and washed specimen stains very evenly with the hematoxylin used.

As a last step preparatory to staining, specimens are placed in a mixture of water, alcohol, and glycerin comparable in concentration with that used in the stain. This pre-soaking reduces the likelihood that the object will float on the surface of the stain and dry out. It also results in more even staining throughout the whole culture area. While the object is soaking in this solution, it is very convenient to hold it down by means of a disk of plastic (not metal) window screening.

Choice of Stain; Staining

The stain used is a ferrous-ferric hematoxylin, further modified from the formula described by Lillie and Earle (*5*). While the stain as originally described was satisfactory for use with sections, when used on tissue cultures its action was erratic. This was found to be due to the lack of control over the degree of "ripening" of the stain. The present method has resulted in much more reproducible preparations.

No attempt has been made to follow in detail the chemical changes in the staining solution. Presumably, the hematoxylin unites with the iron to form a lake, the excessive oxidation of which is retarded by the addition of the ferrous compound and by later addition of glycerin and alcohol. That some oxidation of the hematoxylin is necessary is indicated by the fact that unless the A-and-B mixture is allowed to ripen for 2 hours before adding C and D, the ripening of the final solution to a stage satsifactory for whole mount tissue cultures takes from 1 to 2 weeks. That the hematoxylin itself is partially oxidized is also indicated by the fact that a usable but slightly overripened stain has been prepared by substituting hematein for hematoxylin and eliminating the 2-hour ripening.

When 1.0 gm. of hematoxylin was used, as called for in the final working formula of Lillie and Earle (*5*), some staining of the thick fibrin clot occurred. This was eliminated by reducing the hematoxylin concentration to 0.9 gm. in the present formula. For sections, the use of the 1.0-gm. concentration probably gives more rapid staining, has better keeping qualities, and is better suited for work with such thin preparations. For further consideration of the role of the different constituents of the stain, reference is made to the preceding articles (*4*, *5*, *7*).

No appreciable fading of the stain has been noted in mounts several years old. No studies have been made relative to varying the composition or manner of use of the stain to show cell structure other than that of the nucleus. Such studies might well be tried, however, since the variation of concentration of hematoxylin and ferric iron gives an extensive range of staining potentialities. For more difficult staining structures, it might be well to explore particularly the activity of higher concentrations of hematoxylin, but these probably cannot be used with thick plasma clots.

In trying a number of other stains on these thick tissue-culture preparations, a number of difficulties were encountered: (1) The stain was often not sufficiently selective and diffusely stained the fibrin clot. This is the fault of all the counterstains tried and holds for stains of more limited selectivity, such as the aluminum hematoxylins. (2) The color value of the stain proved optically difficult to study, as exemplified in the red carmine stains. (3) The stains could not be evenly differentiated. This trouble has been routinely experienced with the various modifications of the usual iron hematoxylins, which must be differentiated in some ac-

[12] On roller-tube cultures of the usual type, the thinner layer of solid-culture medium used would probably necessitate substantially fewer changes of the fixing solution to establish a satisfactory pH level.

tive differentiating solution, such as iron-alum solution. With the use of such solutions, differentiation of the cells at and close to the surface of the fibrin clot leaves the cells and matrix within the preparation too deeply stained, whereas when the process is carried sufficiently far to differentiate the interior of the object, the cells at the surface are too lightly stained. The staining solution described herein seems better in this respect than any so far tried. Its applicability to other types of thick preparations where cell identification or nuclear structure is desired, for instance in whole mounts of young embryos, seems worth exploring. This stain also has an optical translucence that is satisfactory in both visual and photographic work. Its color ranges from deep blue to a deep blue-black.

During staining and washing the introduction of metal objects into the stain or wash solution seems to injure the stain.

Dehydration and Clearing

In the earlier preparations, substantial difficulty was experienced in dehydration in alcohol and clearing (in benzene, toluene, or xylene) because of extreme shrinkage of the object. Practically all this shrinkage occurred in the transition from alcohol to toluene (benzene or xylene). It apparently cannot be eliminated or substantially reduced by effecting the transition from alcohol to toluene by even very closely graded stages. Shrinkage was greatly increased on damp summer days, and it was finally found that it could be reduced or almost eliminated by using nearly anhydrous alcohol. A concentration of water in the absolute alcohol, which caused no visible cloud when toluene was added, could nevertheless cause very severe shrinkage when the object was transferred to toluene. One instance may be cited. On changing a strip of fibrin clot 23 mm. long from water-containing absolute alcohol to toluene, the strip shortened to 15 mm. within 30 minutes.[13] When chemically pure acetone was used instead of alcohol, shrinkage was negligibly slight. Substitution of acetone for the alcohol was found to be hazardous, however, since it increased the chance that the specimen would dry during the manipulations in changing from one concentration of acetone to the other, particularly in the higher acetone concentrations. Since toluene evaporates less readily than acetone, the difficulty was finally eliminated by carrying the specimen up through absolute alcohol, then through the series of clearing mixtures described previously. The use of these mixtures made possible the routine dehydration of the preparation by alcohol and its final transfer over to toluene (or xylene) with a minimum of shrinkage or deformation. An actual test of the shrinkage during this process showed that a test fibrin strip shrank from 22 to a final length of 21 mm.

[13] Such fibrin strips cut from the clot of flask cultures fixed as described have proved most sensitive shrinkage-test objects. A similar test-fibrin strip was used in a study of the action of Bouin's solution. In this study, the test strip shrank in about 1 hour from a size of 20 x 10 mm. to 15 x 7.5 mm. during fixation.

Mounting

Clarite as a mountant for these preparations has been found much superior to balsam in that it is practically colorless and is much more inert in its influence on the stain.

The drying of the mounted preparation before adding the lead weights of the cover slip allows a preliminary hardening of the clarite which prevents the lead weights from squeezing the paper chamber out from under the cover slip. It also allows the evaporation of much of the solvent from the thick layer of clarite, while the cover slip is floating free on the clarite. Elimination of the solvent tends to pull the cover slip down closer to the slide instead of sucking air into the cell, as would be the case if the cover slip were already resting solidly on the paper cell. Such preliminary drying may be carried out with the slide level on a slide warmer or in an incubator set at 40° to 45° C.

Examination of Preparation

The final preparations are about 0.45 mm. thick measured from the top of the slide to the top of the No. 1 cover slip. This thickness allows examination of the whole thickness of the culture with either an 8-mm. apochromatic objective, a 5.5-mm., 0.65 N. A. achromatic objective, or a 4-mm., 0.65 N. A. achromatic objective. A 3-mm., 1.4 N. A. apochromatic objective is practically limited to the uppermost sheet of cells of the culture; but since most of the cells are at the glass and fluid interfaces in the culture and since the culture is split in half (the two halves being mounted with different faces up), even with this high resolution objective, a large percentage of the cells in the culture may be studied. For most detailed high-power work with these preparations, the author prefers the 3- or 4-mm. immersion objective of 0.85 to 1.0 N. A., usually sold for use with the dark-field condenser. When used with 15× compensating oculars, its magnification and resolution are adequate to handle any structure which may reasonably be studied in such whole-mount preparations, while the free working distance of 0.22 mm. (with the Zeiss 3-mm., 1.0 N. A. immersion Apochromat) to 0.27 mm. (with the Bausch & Lomb 4.3-mm. fluorite oil-immersion objective [14]) allows the examination of cells practically half way down through the preparation. Below this depth, the diffusion of

[14] Information has just been received that this objective can be obtained on special order with a free working distance of 0.5 mm.

light by the clot interferes with the perfection of the image. When an achromatic objective is used, because of its longer free-working distance as compared with that of the apochromat, the optical image may be made considerably clearer through elimination of color fringes by using such a filter as the green Wratten B (No. 58) or the yellow Wratten G (No. 15) in the light beam. The latter is frequently used with these stained preparations.

In examination of such stained preparations a light source comparable in intensity with the 6-volt, 108-watt, ribbon-filament microscope research lamp, adjusted for critical illumination, is satisfactory; although for some more densely cellular areas a more intense source of light, such as the enclosed tungsten-bead arc, is often a great help.

When hanging-drop cover-slip preparations of cells growing in tissue culture are studied in order to obtain a maximum of cell detail with high resolution lenses, the general procedure of fixation and staining outlined gives extremely satisfactory results.

SUMMARY

A method is described for making whole-mount preparations of tissue cultures, grown in Carrel D 3.5 flasks. The method involves fixation in formalin-potassium dichromate solution of controlled pH, hardening in 3 percent potassium dichromate, staining in a ferrous-ferric hematoxylin solution specially modified for staining thick preparations, dehydrating in alcohols, clearing in special mixtures of alcohol, acetone, and toluene, and mounting in paper cells in clarite. Slides are seasoned at about 40° C. for about 60 days, the cover glasses meanwhile being pressed down by lead weights.

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CUBAN CANCER CONGRESS

The Third National Cancer Congress under the auspices of the Governor of the Republic, the Cuban Society of Cancer, and the League Against Cancer will be held February 23 to 28, 1948, in Havana. Participation is invited. Inquiries should be directed to Comite Organizador, Oficinas de la Liga contra Cancer, Calle F esq. a 29, Vedado, Habana, Cuba.